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13. SUPPLEMENTARY NOTES

14. ABSTRACT- Neurobiological research has implicated abnormalities in medial prefrontal cortical function (mPFC), as well as altered hypothalamus-pituitary-adrenal (HPA) axis function in Post-traumatic stress disorder, but the mechanisms that link PTSD symptom generation, mPFC medial function, and stress axis abnormalities have not been established. PTSD's clinical manifestation includes intrusive recollections of the traumatic event, avoidance of social interactions, and a failure to regulate fear and anxiety. We used the Single Prolonged Stress (SPS) model to examine the effect of SPS on HPA and mPFC function and how this relates to specific PTSD symptoms. Our data suggest that SPS animals have disruptions in the retention of extinction memories, and that augmentation of GR expression in the PFC and hippocampus is linked to SPS-induced extinction retention deficits. SPS animals also have decreased levels of glutamate in the mPFC, suggesting decreased excitatory neurotransmission in a brain region critical for emotion regulation. In addition, we have also demonstrated enhanced noradrenergic reactivity in the locus coeruleus of SPS rats using electrophysiological recording and TH mRNA levels. Preliminary findings relating to social interaction in SPS animals have not been robust or consistent, but we have demonstrated that some SPS effects can be attenuated by increasing mother-pup social interactions, and that inactivation of the BLA attenuates social interactions. We also found that SPS impairs another form of behavioral regulation, cognitive flexibility. During the funding period we have made significant advances in understanding neurobiological responses to trauma, linking specific behavioral changes to changes in HPA axis, and finding evidence of changes in glutamatergic transmission and noradrenergic system activity. These findings have filled a considerable gap in our scientific understanding of the biological basis of PTSD, and provide the necessary foundation for future research both into exact mechanisms of symptom development and into the development of novel strategies aimed at treatment and prevention of the specific PTSD symptomatology.

15. SUBJECT TERMS- PTSD, SPS, anxiety, fear, conditioning, prefrontal cortex, hippocampus, amygdala

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Introduction

The aim of this research program was to investigate stress induced changes in neural processes that lead to aberrant psychological responses/behaviors that mimic PTSD symptomatology. PTSD's clinical manifestation includes, amongst others, three key sets of symptoms that lead to significant disability and poor overall functioning: the recurrent and intrusive recollections of the traumatic event, avoidance of normal social interactions, and the perception that emotions like fear, anger, and anxiety are beyond the control of the patient (i.e. deficit in regulation of aversive emotions). Neurobiological research has implicated abnormalities in medial prefrontal cortical function centrally, as well as altered hypothalamus-pituitary-adrenal (HPA) axis function in PTSD; however, the mechanisms that link PTSD symptom generation, medial prefrontal cortical function, and stress axis abnormalities have not been established. In the last decade our laboratory has developed an animal model of PTSD that induces PTSD specific, HPA axis changes, and behavioral arousal characteristic of PTSD in rodents: Single Prolonged Stress (SPS). Our preliminary data suggested that SPS induced deficits in extinction, avoidance of social interactions, and deficits in modulation of aversive responses (emotional regulation). These findings offered an outstanding opportunity to study potential neurobiological mechanisms involved in the generation of a key set of PTSD symptoms in a validated animal model. Our general hypothesis was that SPS-induced deficits in these PTSD-like symptoms were caused by the effect SPS has on HPA and medial prefrontal cortical (mPFC) function. We proposed to test this general hypothesis by evaluating the following specific aims: #1): Examine the roles of altered mPFC function and expression of brain glucocorticoid receptors in the development of SPS induced extinction deficits (as a model of PTSD intrusive symptom cluster). # 2): Examine the roles of altered mPFC/amygdala function and of HPA/glucocorticoid receptor (GR) function in the development of SPS induced avoidance of social interactions (as a model of PTSD social avoidance cluster). #3): Examine the role of altered mPFC/amygdala function and of altered HPA/GR function in the development of SPS induced deficits in defensive behavior regulation (as a model of emotional dysfunction in PTSD). # 4): Examine the ability of Selective Serotonin Reuptake Inhibitors (SSRIs) and anti-kindling drug administration to alleviate SPS induced extinction deficit, social avoidance, and defensive behavior regulation deficits; and the role of mPFC/amygdala function and HPA/GR function in this process. In our first year of funding our goal was to replicate our preliminary findings and then proceed with testing of the hypotheses in the specific aims. We conducted a large number of studies to determine the effect of SPS on fear conditioning and extinction and found that SPS produces a specific deficit in the recall of fear extinction, while leaving the acquisition of fear conditioning and fear extinction intact. We did replicate consistently the preliminary findings of SPS effects on social interaction test and defense behavior regulation. However, we did find additional behavioral deficits in SPS animals as we further discuss below. As a result, in our second year of funding we proposed a revised statement of work that was accepted by the agency, and continued with testing of the specific aims. We believe that the data included in this report, and the number of peer reviewed publications and conference abstracts that this work has led to, demonstrate high productivity and substantial progress in our understanding of neurobiological consequences of traumatic exposure, that resulted from our work. We have made significant advances using SPS towards understanding the mechanisms that link PTSD symptom generation, medial prefrontal cortical function, and stress axis abnormalities.

In this final report we detail our key research accomplishments for the entire funding period (9/1/08 – 8/31/12). All publications (peer reviewed publications and conference abstracts) are listed in the Reportable Outcomes section. The revised Statement of Work that was approved by the review committee is presented in Appendix I. Personnel paid on this grant are listed in Appendix II, and all publications resulting from this grant are in Appendix III.

Body

The effect of SPS on fear conditioning, extinction, extinction recall and renewal.

In our preliminary data section for our application we presented data that suggested that SPS disrupted cued fear extinction. Prior to embarking on the experiments to address specific aim #1, we planned to replicate this finding and describe these findings in more details i.e. what specific phase of fear extinction was disrupted. After conducting repeated experiments, we determined that SPS induces specifically deficits in cued extinction retention/recall, while leaving cued fear conditioning and extinction acquisition intact. Given these findings we further explored the effect of SPS on difference aspects of contextual and cued conditioning in detail. The results of these studies determined that; 1) SPS induced deficits in contextual extinction recall (Figure 1), SPS disrupted SPS cued extinction recall and enhanced fear renewal (Figure 2), and the effect of SPS on extinction recall requires the seven day post-stress quiescent period (Figure 3). These experiments had direct relevance to evaluating specific aim #1. Detailed methods, results and statistical analyses can be found in Knox et al. (2012a) and attached in Appendix III).

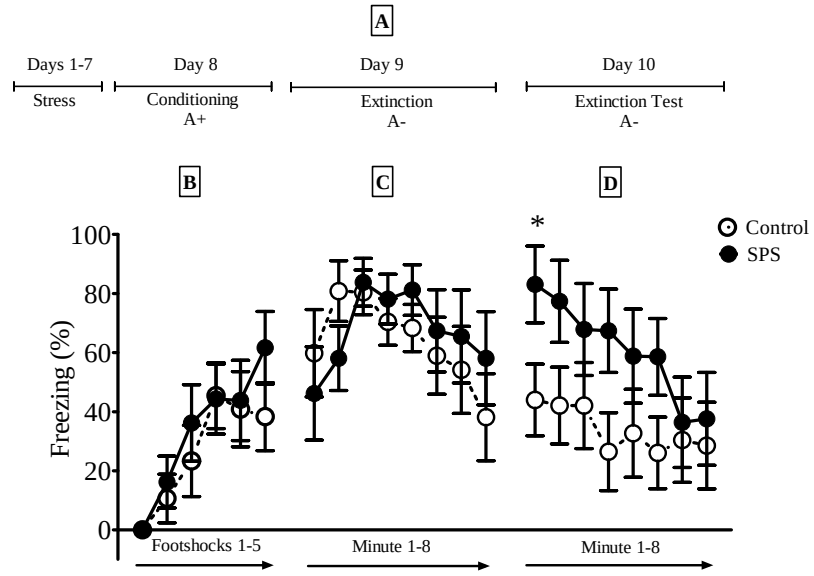

Figure 1. SPS induced deficits in contextual extinction retrieval. (A) Diagram illustrates experimental design used in this study. The two character (e.g. A+) symbol describes conditioning and extinction parameters. First letter denotes context and second character denotes the presence or absence of footshocks. (B) SPS had no effect on freezing during conditioning or (C) extinction, (D) but disrupted contextual extinction retrieval.

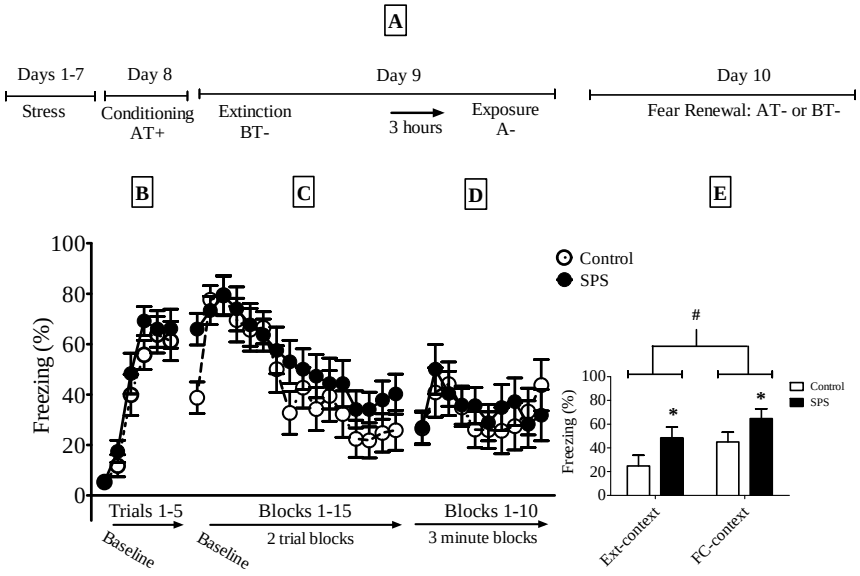

Figure 2. SPS disrupted cued extinction recall and enhanced fear renewal. (A) Illustrates the experimental design used in this study. T denotes tone presentation. (B) SPS had no effect on freezing during conditioning (C) extinction, or (D) re-exposure to the conditioning context. (E) SPS disrupted extinction recall irrespective of the context in which extinction was tested (i.e. disrupted extinction recall and enhanced fear renewal).

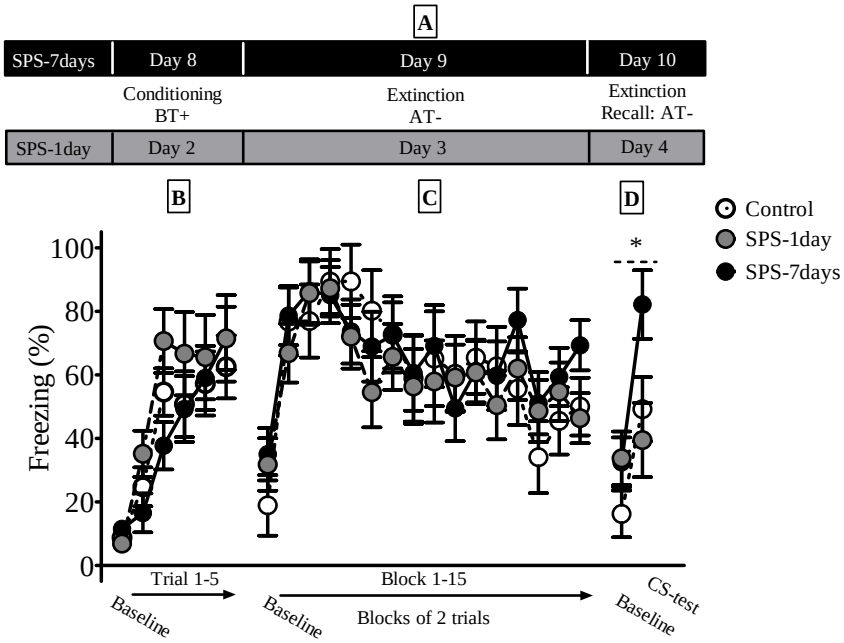

Figure 3. The effect of stress on extinction recall requires a post-stress incubation period. (A) Illustrates the experimental design used in this experiment. (B) Neither the SPS-1 day nor SPS-7 days rats displayed different freezing levels during conditioning or (C) extinction. (D) Extinction recall was impaired in the SPS-7 days group, but not SPS-1 day group.

SPS-enhanced fear renewal is mediated by decreases in neural activity in the hippocampus and BLA.

In order to determine which neural substrates are critical for SPS-induced deficits in extinction recall/renewal (specific aim #1b), we collected brains from the rats in the above study and measured c-fos mRNA as a marker of neural activity. c-fos is an immediate early gene that is upregulated when there is increased neural activity. We measured c-fos expression in regions of the mPFC (infralimbic (IL) and prelimbic (PL) regions), hippocampus, basolateral nucleus of the amygdala (BLA), and locus coeruleus (LC). While we initially proposed to examine the effects of SPS on neural activity using single unit electrophysiology, c-fos has the advantage in that you can examine neural activity in several brain regions simultaneously. These data are illustrated in Figure 4. We did not find any difference in c-fos expression between SPS and control rats during extinction recall (Figure 4c), but we did find decreased c-fos expression in the BLA and hippocampus of SPS rats during fear renewal (Figure 4c), suggesting that SPS may enhance fear renewal by decreasing neural activity in the BLA and hippocampus.

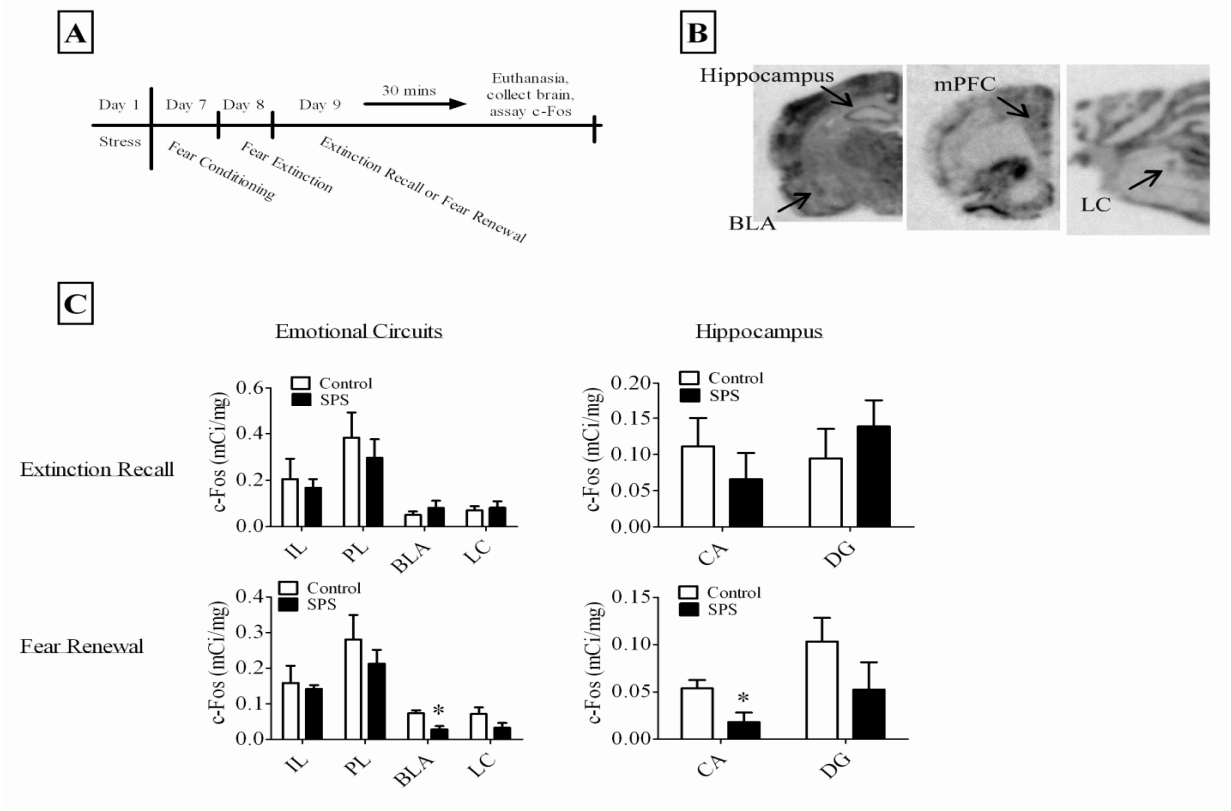

Figure 4. The experimental design for this experiment is illustrated in Figure 4a. Figure Example of c-fos expression. There were no difference in c-fos expression between SPS and control rats during extinction recall (Figure 4c), but we did find decreased c-fos expression in the BLA and hippocampus of SPS rats during fear renewal (Figure 4c).

SPS-enhanced GR expression in the hippocampus and PFC contributes to SPS-induced extinction recall deficits.

One of the main goals of specific aim #1 was to determine the relationship between SPS-enhanced GR expression and SPS-induced extinction recall deficits. We have recently demonstrated that SPS-induced changes in the retention of extinguished fear is linked to upregulated GR expression in the hippocampus and PFC (see Appendix III, Knox *et al.*, 2012c). In this experiment, we compared the effect of "partial SPS" – consisting of just two of the three SPS stressors – to full SPS and control groups. We found that exposing animals to partial SPS in this manner abolished stress-induced extinction retention deficits and significantly attenuated stress-enhanced GR expression in the hippocampus and PFC. These findings suggest that full SPS exposure is required to produce behavioral changes and that enhanced GR expression is critical for expression of SPS-induced extinction retention deficits. Figure 5 shows a summary of these findings.

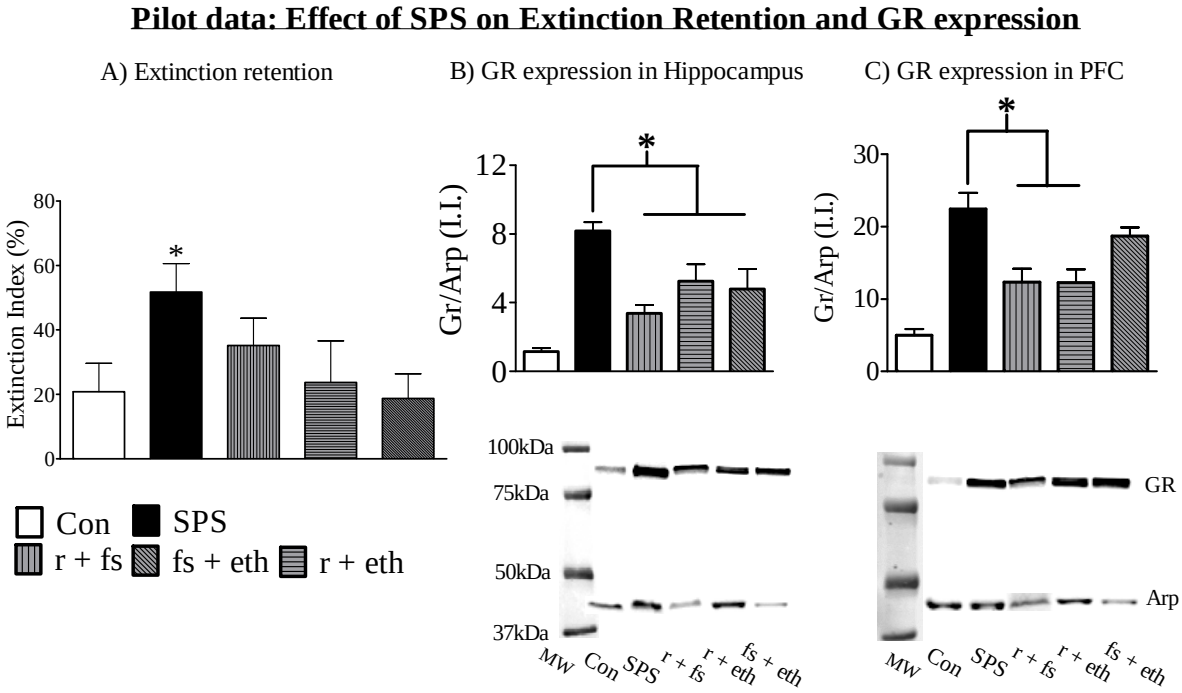

Figure 5. Effect of SPS or partial SPS (p-SPS; two of the three SPS stressors) on extinction retention (A) and GR expression in the hippocampus (B) and PFC (C). Only SPS induced extinction retention deficits, and while exposure to SPS and all p-SPSs enhanced GR expression in the PFC and hippocampus, SPS-enhanced GR expression was significantly greater following SPS. *p<0.05.

The Effect Of SPS On Glutamate Concentrations In The Medial Prefrontal Cortex, Hippocampus, And Amygdala

Given the effect of SPS on extinction and extinction recall, and the importance of the medial prefrontal cortex, hippocampus, and amygdala in these processes, we examined the effect of SPS on inhibitory and excitatory tone in the medial prefrontal cortex, hippocampus, and amygdala by assaying basal glutamate and GABA concentrations. We did this by using high-resolution magic angle spinning proton magnetic resonance spectroscopy (MRS). Extending our original line of research in this direction addressed possible neurobiological mechanisms in mPFC and hippocampus involved in SPS effects in fear extinction and extinction recall, which directly relates to specific aim #1. We found that the concentration of glutamate was attenuated in the mPFC of SPS rats when compared to controls, while the concentration of succinate in the mPFC was equivalent for SPS and control rats, which suggests that the changes in the concentration of glutamate were restricted to glutamate used in neural transmission. In addition, the concentration of glutamine (a major precursor molecule for synthesis of neuronal glutamate) (Nicholls, 1994) was also attenuated in the mPFC. The concentration of GABA was not different for SPS and control rats in the mPFC, which also suggests SPS-induced decreases in glutamate in the mPFC was restricted to glutamate used for neural transmission because glutamate is a precursor molecule for the synthesis of GABA (Cooper *et al.*, 1996). There was no change in glutamate or GABA in the hippocampus or amygdala complex. These results are illustrated in Figure 6 and contained in Appendix III (Knox *et al.*, 2010).

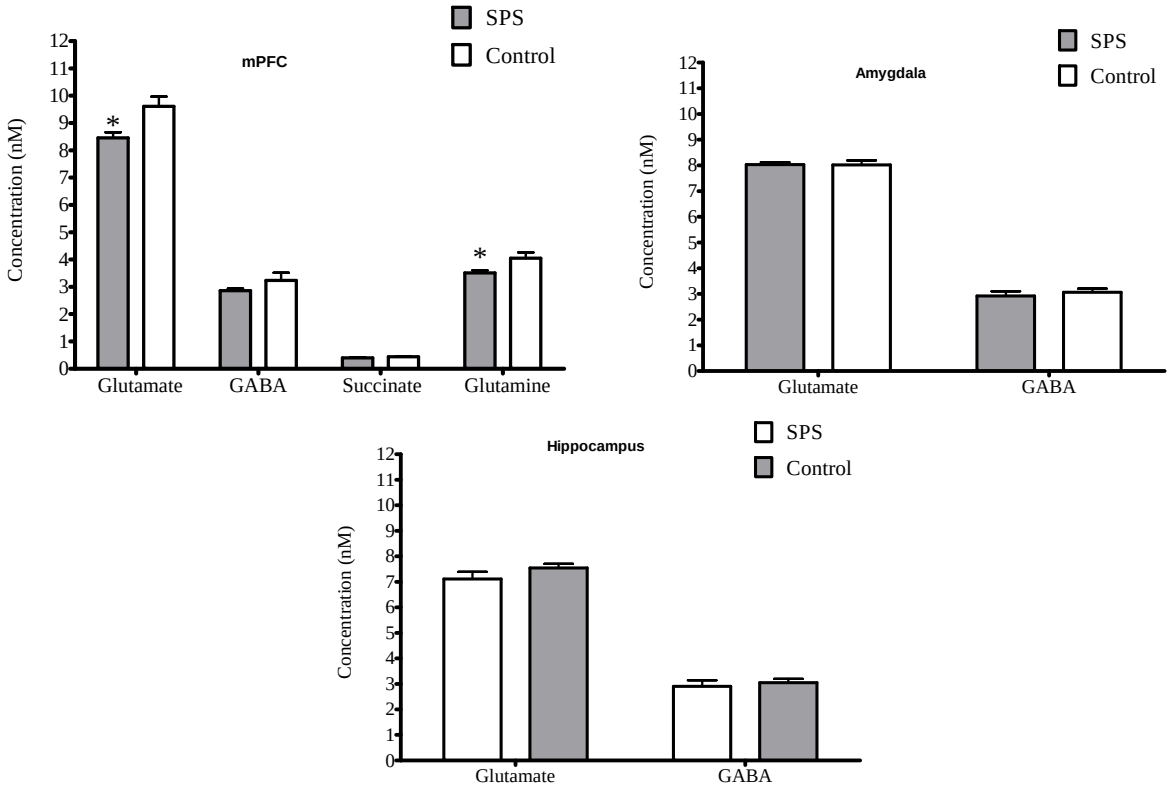

Figure 6. SPS reduced glutamate, glutamine and succinate concentrations in the mPFC but did not alter glutamate or GABA in the amygdala or hippocampus.

The Effect Of SPS On Social Avoidance In The Social Interaction Test.

Our second piece of preliminary data involved the demonstration that SPS impaired interactions in the social interactions test, and formed the basis of our specific aim #2. However, after extensive testing we were unable to replicate this finding [p = 0.77]. These results are illustrated in Figure 7.

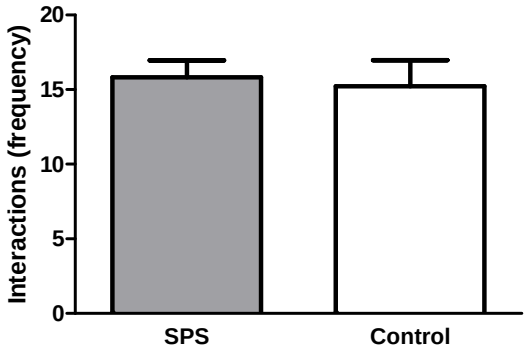

Figure 7. SPS did not alter the frequency of social interactions

Given that we could not replicate our previous findings with regard to the SPS effects on social interaction we continued to address the hypotheses stated in Specific Aim #2 by exploring whether SPS rats are resistant to the positive effects of social interactions in a number of different paradigms such as mother-pup social interactions, and social buffering (hypotheses #2c and #2d).

The Effect Of SPS On Social Buffering

Social buffering refers to attenuated expression of fear conditioned freezing when two rats are simultaneously evaluated for fear conditioned freezing. Rats were assigned to SPS or control conditions and fear conditioning, extinction and extinction recall was conducted. During fear extinction, both partner and experimental rats were placed into the fear conditioning context and tones were presented. Freezing to each tone was then documented. Both SPS and control rats demonstrated robust fear conditioned freezing. There was a paradoxical stress effect with SPS rats exhibiting lower freezing in the presence of a partner rat than control rats. Given that it did not appear any buffering took place in the control rats and the variability in the data, these data were difficult to interpret and another method of assessing social buffering was sought.

The protective effect of mother-pup social interactions attenuates some, but not all, SPS behavioral and physiological effects.

In this experiment, mother-pup social interactions were enhanced by briefly separating pups from their mother over a three week period during the neonatal phase of development.

Previous research has demonstrated that this enhances mother-pup social interactions (Cirulli *et al.*, 2007) and reduces anxiety behavior and stress when pups become adults (Lehmann & Feldon, 2000). We then subjected rats to either SPS or control procedures, and evaluated startle reactivity in all rats. After this, brains were extracted and GR expression assayed in the PFC and hippocampus. SPS enhanced startle reactivity and GR expression in the PFC and hippocampus. Rats that had enhanced mother-pup social interactions when they were neonates were protected against the effects of SPS on startle reactivity and GR expression in the PFC. However, SPS-induced changes in GR expression in the hippocampus were unaffected by enhanced mother-pup social interactions. These results are illustrated in Figure 8, and suggest social buffering during the neonatal phase of development can reduce some, but not all, SPS-induced effects.

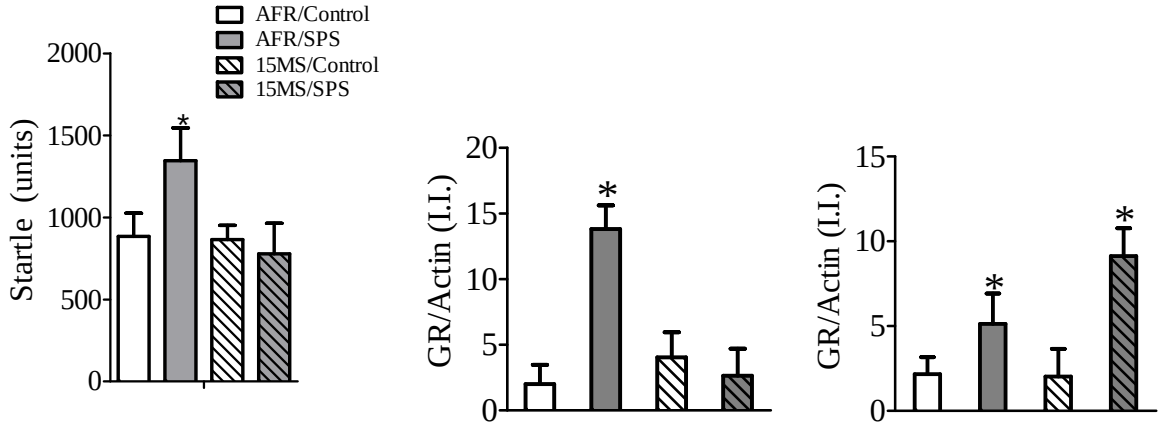

Figure 8. The effect of enhancing maternal-pup social interactions on SPS effects. Enhancing maternal-pup social interactions attenuated the A) SPS-enhanced startle reactivity and B) GR expression in the prefrontal cortex. However, enhancing maternal-pup social interactions had no effect on C) SPS-enhanced GR expression in the hippocampus. GR in the prefrontal cortex and hippocampus were assayed using western blot electrophoresis, and expressed as a ratio increase over levels of actin (i.e. GR/Actin ratio). I.I. – integrated intensity of a protein band. AFR – animal facility reared (i.e. a control for the 15MS experimental group), 15MS – rats treated so as to enhance mother-pup social interactions.

Inactivation of the BLA attenuates social interactions

In Specific Aim #2, our goal was to determine the role of the BLA in social interactions. The BLA is critical for expression of fear (Sotres-Bayon *et al.*, 2004), and thus we hypothesized that inactivation of the BLA would enhance social interactions. In order to test this, we temporarily inactivated the BLA and documented the effect this had on social interactions in the social interaction test (File & Seth, 2003). As shown in Figure 9, BLA inactivation *decreased* social interactions. This suggests that the BLA is critical for the generation of social interactions. However, the results could also mean that the BLA is critical for inhibiting anxiety during social encounters. In order to rule out this alternative hypothesis, we conducted another experiment in which we examined the effect of BLA inactivation on anxiety behavior in the elevated plus maze. In this test, BLA inactivation had no effect on anxiety behavior. This is illustrated in Figure 10. Thus, the results of these studies together suggest that the BLA is critical for generating social behavior.

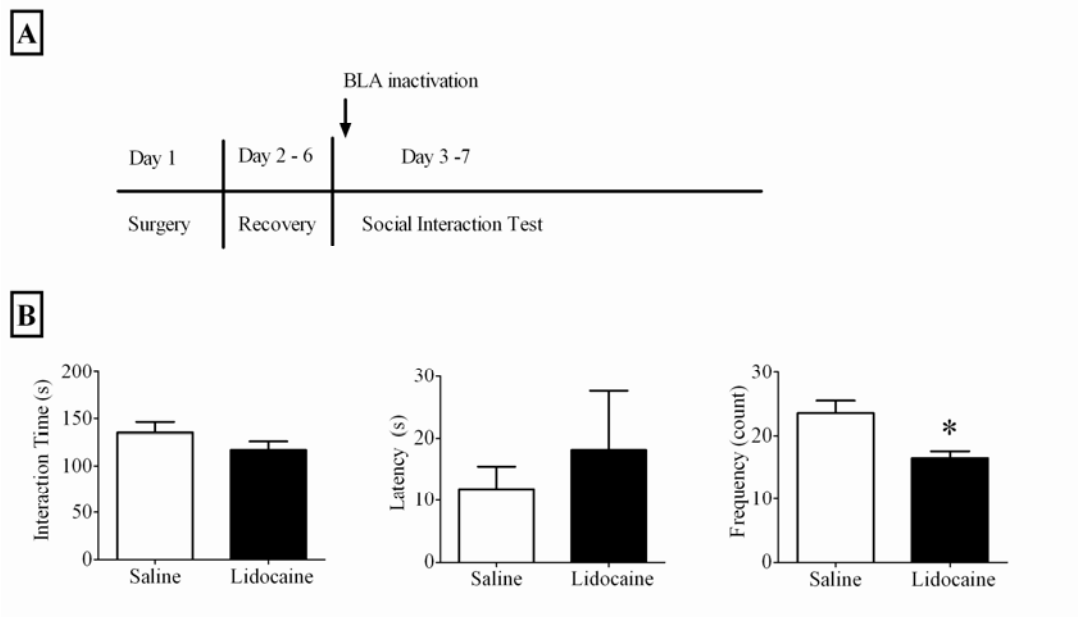

Figure 9. A) Experimental design for this study. B (left panel) Inactivation of the BLA had no effect on social interaction time, (middle panel) latency to the first social interaction, but (right panel) attenuated the number of interactions in the social interaction test.

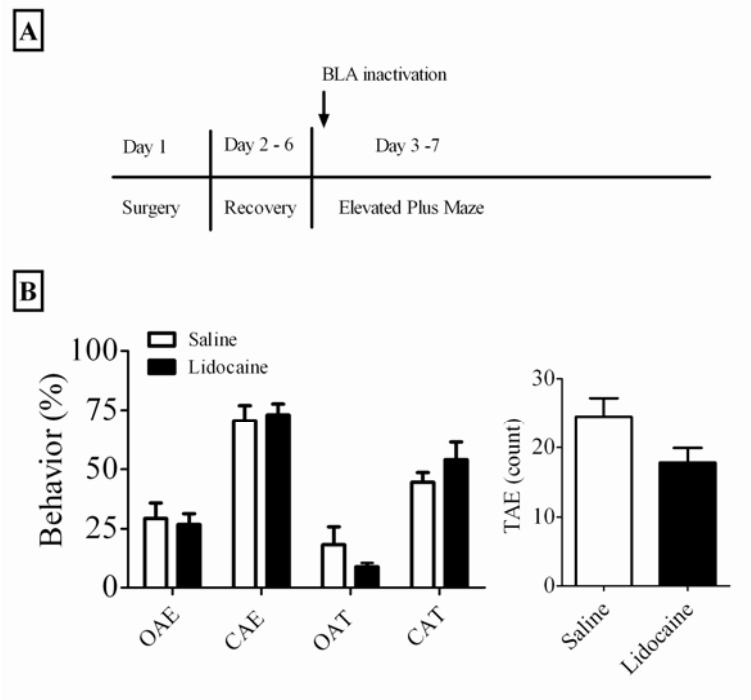

Figure 10. A) Experimental design for this study. B left panel) Inactivation of the BLA had no effect on anxiety behavior or (right panel) total arm entries (locomotor activity metric) in the elevated plus maze. OAE – open arm entry, CAE – closed arm entry, OAT – open arm time, CAT – closed arm time, TAE – total arm entry.

The Effect Of SPS On Defense Behavior Regulation

In our grant proposal we presented preliminary data that suggested prior exposure to an appetitive context attenuates trimethylthiazoline (TMT)-induced freezing in control rats, but not SPS rats. We interpreted this finding to mean that freezing behavior is decreased in an appetitive context (i.e. defense behavior regulation) and this process is disrupted in SPS rats. These findings were not replicated however in a larger subsequent study (Figure 11). All rats displayed a progressive increase in freezing upon presentation of TMT. This was reflected by a significant main effect of time [$F(4,132) = 10.575$, $p < .001$]. However, SPS did not alter this effect [$F(1,68) = 1.10$, $p = .298$]. However, from the studies conducted to address this specific aim, we obtained a number of significant findings that nonetheless have relevance to PTSD. These are described below.

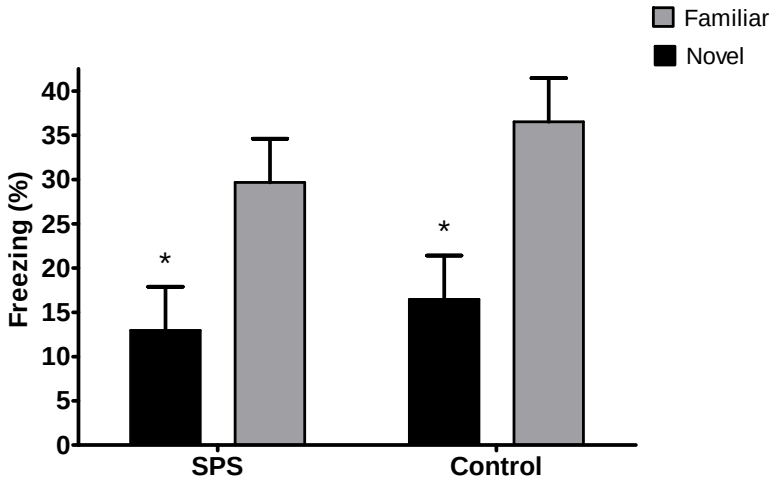

Figure 11. SPS effects on TMT-induced freezing.

Unconditioned fear is enhanced in an appetitive context and is mediated by deactivation of the mPFC

We explored the effect of infralimbic cortex (IL) and prelimbic cortex (PL) inactivation on TMT-induced freezing. While there is a lot of research investigating the role of the mPFC in conditioned fear and extinction, the role of these brain regions in unconditioned fear has not been well examined. In addition, in human clinical practice it is recognized that the expression of fear is modulated by the context in which fear is evoked and while many animal reports have demonstrated that extinction is modulated by context, the role in the expression of unconditioned fear has not been determined. To address hypothesis #3a, rats were equipped with guide cannulas aimed at either the PL or IL. These regions were then temporarily inactivated prior to TMT-induced freezing and the effect of inactivation documented. The results of this experiment demonstrated that 1) the prelimbic cortex is critical for inhibition of unconditioned freezing (see Figure 12). Having demonstrated that PL inactivation enhances TMT-induced freezing, we wanted to see if enhanced TMT-induced freezing in an appetitive context results from deactivation of neural activity in the PL and enhanced neural activity in the amygdala. Rats were

tested for TMT-induced freezing in a novel, familiar, or appetitive context. Thirty minutes after the start of the test, rats were euthanized, brains extracted, and c-fos mRNA (used to measure neural activity) assayed in the mPFC and amygdala. TMT-induced freezing was enhanced in the appetitive context. This enhancement was accompanied by decreased neural activity in the PL and IL but not the amygdala. The results of this experiment demonstrated that 2) the expression of unconditioned fear is enhanced in an appetitive context, and 3) enhanced TMT-induced freezing in an appetitive context is mediated by decreased neural activity in the mPFC (see Figure 13 and appendix III, Knox et al. (2012b)).

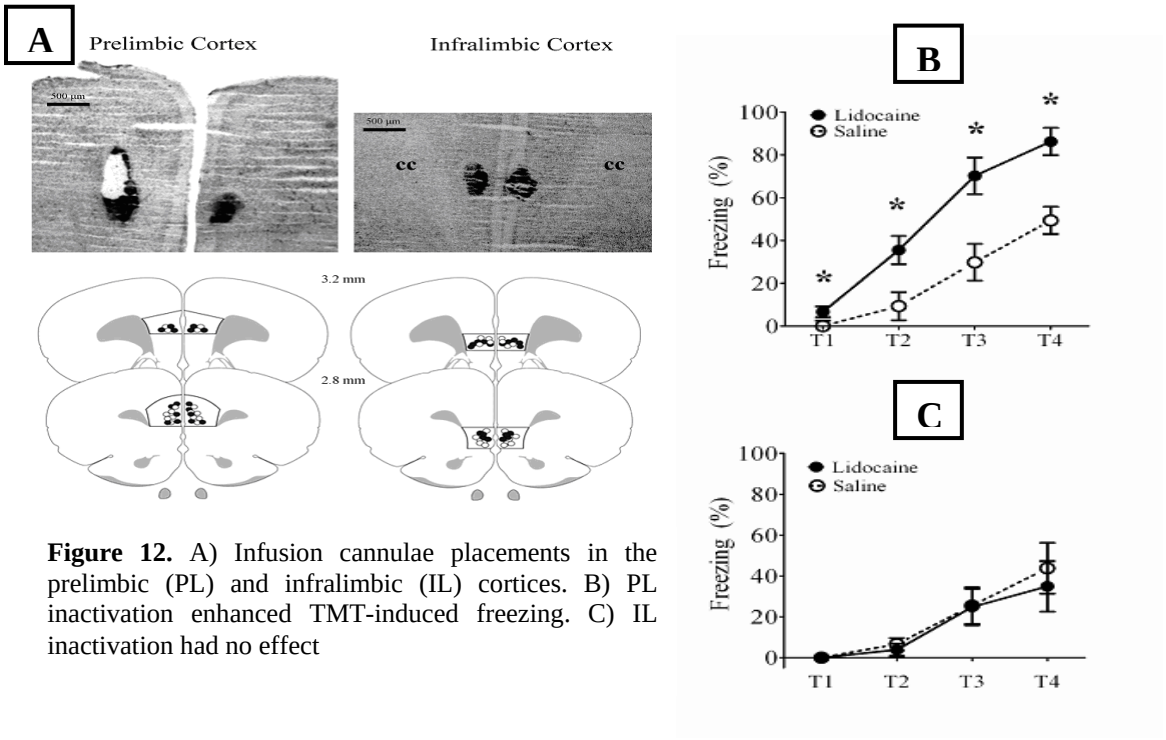

Figure 12. A) Infusion cannulae placements in the prelimbic (PL) and infralimbic (IL) cortices. B) PL inactivation enhanced TMT-induced freezing. C) IL inactivation had no effect

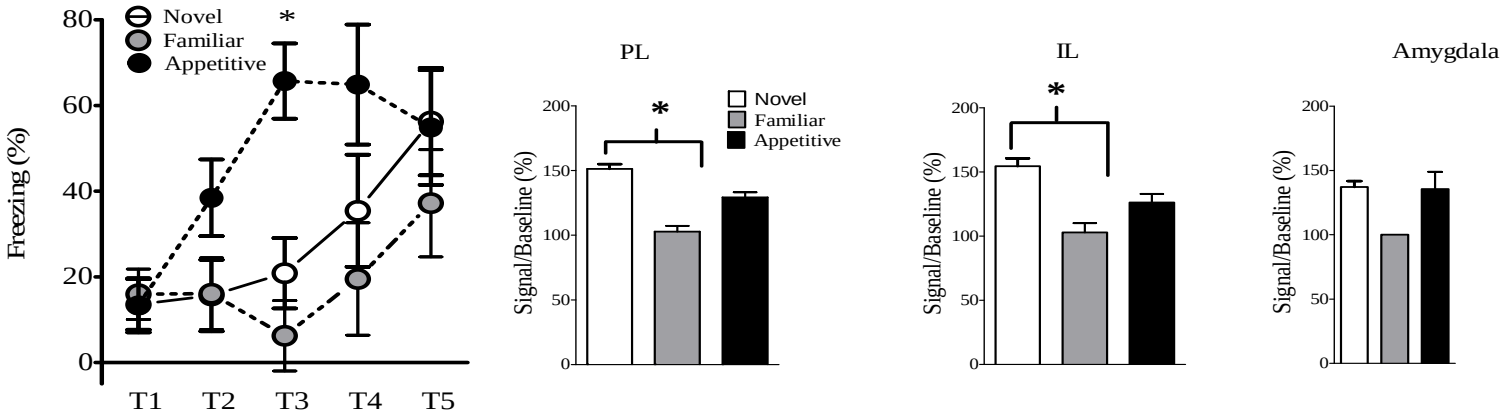

Figure 13. Effect of context on TMT-induced freezing and c-fos mRNA expression. (Left panel) TMT-induced freezing is enhanced in an appetitive context. (Second and third panels) This effect appears to be mediated by decreased neural activity in the PL and IL. Given that only temporary inactivation of the PL enhances TMT-induced freezing, it would appear that decreased neural activity in the PL underlies enhanced TMT-induced freezing in an appetitive context (Last panel) Amygdala neural activity does not drive contextual modulation of unconditioned freezing. C-fos mRNA intensity was scored and expressed as a percent change over baseline.

The Effect Of SPS On Conditioned Emotional Responding and Conditioned Discrimination

In specific aim #3 we were interested in determining the effects of SPS on emotional regulation and designed a novel paradigm in which to test the effects of SPS on emotional regulation (described above). Much of the research we have conducted indeed suggests that SPS disrupts emotional regulation. For example, our findings that SPS induces deficits in extinction recall can be interpreted as a deficit in emotional regulation (Quirk *et al.*, 2006) as it can be argued that SPS rats fail to use extinction memories to regulate levels of fear. Similarly, our findings that SPS enhances fear renewal can reflect the possibility that SPS rats are hypersensitive to contextual inconsistencies between extinction training and testing, and this hypersensitivity then elevates expression of conditioned fear (Bouton *et al.*, 2006). Emotional regulation likely involves the use of a number of other cognitive processes to regulate emotional levels (Campos *et al.*, 2004). To date, there are virtually no established behavioral paradigms that can be used to examine these kinds of cognitive processes in animals. We therefore attempted to examine different related cognitive processes, which we describe below. First, we investigated the effects of SPS on behavioral paradigm that encompasses defense behavior regulation: conditioned emotional responding. In these paradigms rats are required to press a lever for food in the presence of a cue that has previously been paired with an aversive event. In order to continue receiving reward, rats have to suppress their innate defense response (freezing) in order to obtain the reward. This process can be conceptualized as regulation of defense behavior and may be used to model emotional regulatory processes. During fear conditioning, (left panel, Figure 14) we found that while there was a significant main effect of time ($p<0.0001$) there was no interaction involving SPS group for freezing during fear conditioning. There was also a significant main effect of time for freezing during tones ($p<0.0001$) but no interaction involving SPS condition ($p=0.73$). A repeated measures ANOVA (within subjects factor: time; between subjects factor: condition) revealed a main effect of time ($p<0.0001$) but no interaction involving condition ($p=0.171$). During CER test (right panel, Figure 14) All rats showed significant suppression to the tone initially ($p<0.0001$ and $p=0.003$ for presentations 1 and 2 respectively) but not any of the later tone presentations with the exception of tone 6 ($p=0.018$). A repeated measures ANOVA showed a significant main effect of time ($F(6,180)=7.069$, $p<0.0001$) but no main effect of condition (SPS vs. control; $F(1,30)=1.577$, $p=0.219$) or interaction involving condition ($F(6,180)=0.592$, $p<0.739$). These data indicated that all animals suppressed to the tone, extinguished the CER over repeated presentations of the CS and that these effects were not significantly different between groups. The apparent difference between SPS and control animals for suppression to the first presentation of the tone seen in the graph below was not significant ($p=0.125$). We therefore concluded that SPS did not impact conditioned emotional responding, and therefore we did not pursue this line of research further (Figure 14).

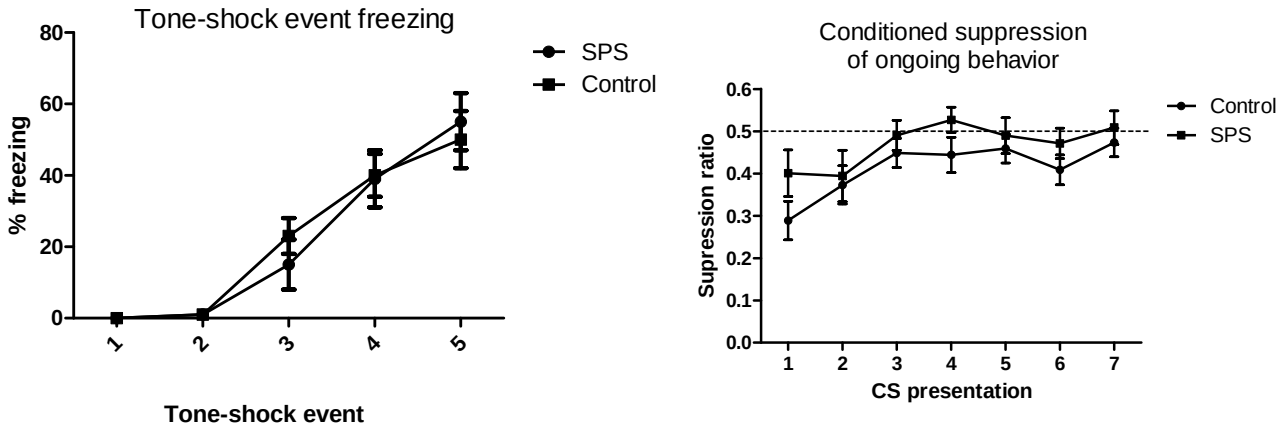

Figure 14. SPS did not alter conditioned suppression of reward motivated behavior

SPS impairs cognitive flexibility

We further examined the effects of SPS on forms of cognitive flexibility, set-shifting and reversal, in which the rats are required to inhibit a previously successful response strategy in order to learn a new response. Deficits in set shifting have been implicated in PTSD and may contribute to emotional regulation problems in this disorder (Walter *et al.*, 2010). In addition, PTSD is associated with neurocognitive impairments that may be attributable to deficits in function of the PFC. Our earlier data demonstrated that rats subjected to SPS show decreased excitatory neurotransmission in the mPFC and impaired regulation of emotional stimuli, but the effect of SPS on other cognitive functions is not known. Rats were trained to press levers, matched for performance and assigned to either SPS or control groups. Following SPS, rats received reminder lever pressing sessions before learning an initial discrimination. For reversal learning rats were trained on a response discrimination strategy on day one, and were given a series of reversals on subsequent days. For set-shifting, animals learned visual cue discrimination and shifted to a response discrimination strategy the following day. Retrieval of the previous day's rule and acquisition of the new rule were tested. SPS and control rats showed comparable performance on acquisition and retrieval of a response discrimination, but SPS rats were impaired on reversal rule [$F(1,29)=4.37, p=.046$]. In contrast, SPS rats exhibited equivalent acquisition, but poorer retrieval of the visual cue discrimination [$t(39)=2.67, p=.011$], and made more 'never-reinforced' errors during the set-shift [$F(1,39)=4.88, p=.033$].

These findings suggest that SPS impairs reversal of simple stimulus-reward associations. It does not affect shifts between strategies, but impairs isolating the most effective strategy after a shift (Figures 15 and 16). These data suggest that SPS induces selective impairments in cognitive flexibility, which disrupt regulation of behavior and are mediated by different regions of the PFC.

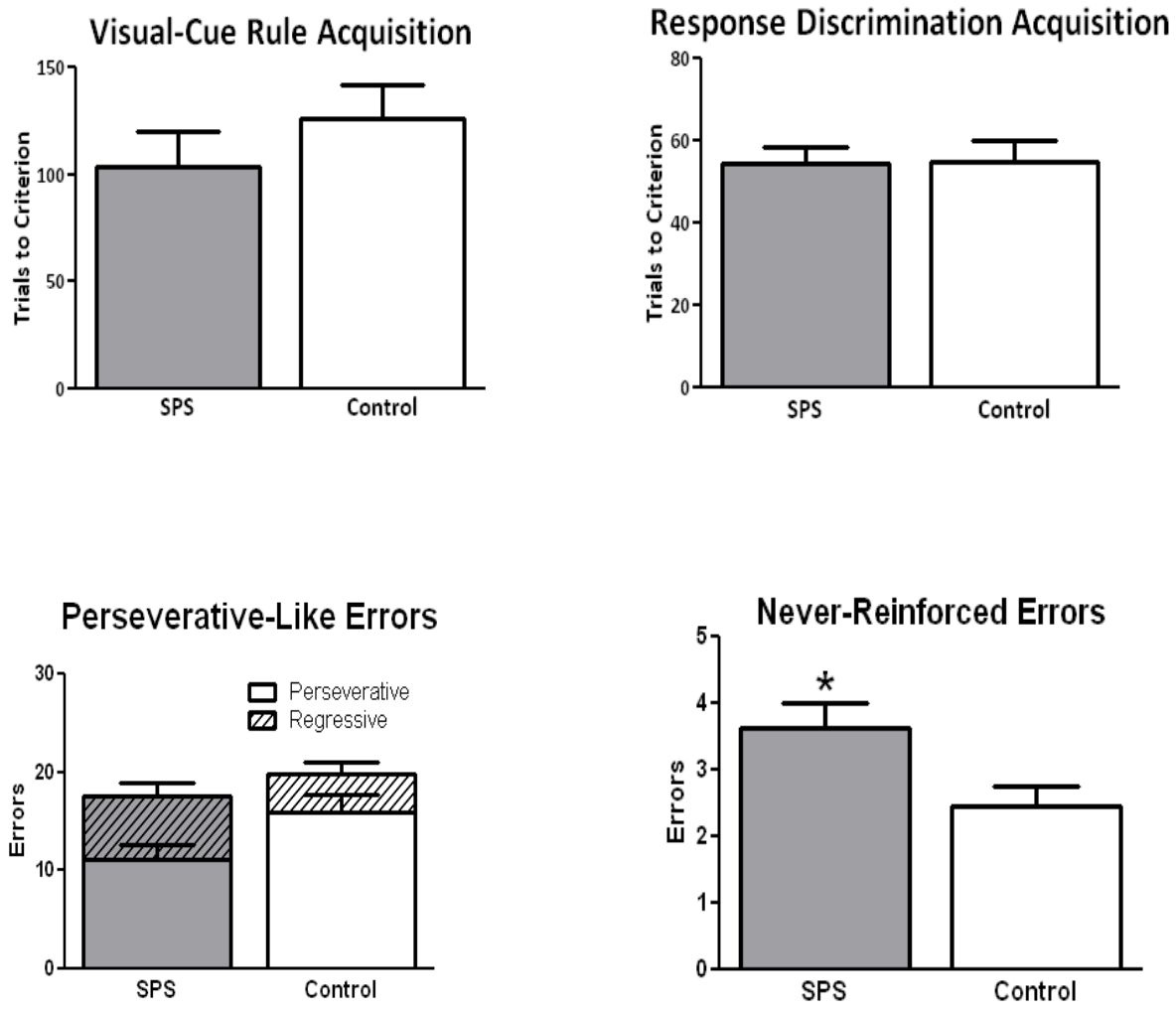

Figure 15. SPS rats displayed unimpaired acquisition of visual-cue and response discrimination rules, there were no difference between SPS and control animals for perseverative or regressive errors, but SPS animals made more never reinforced errors than controls.

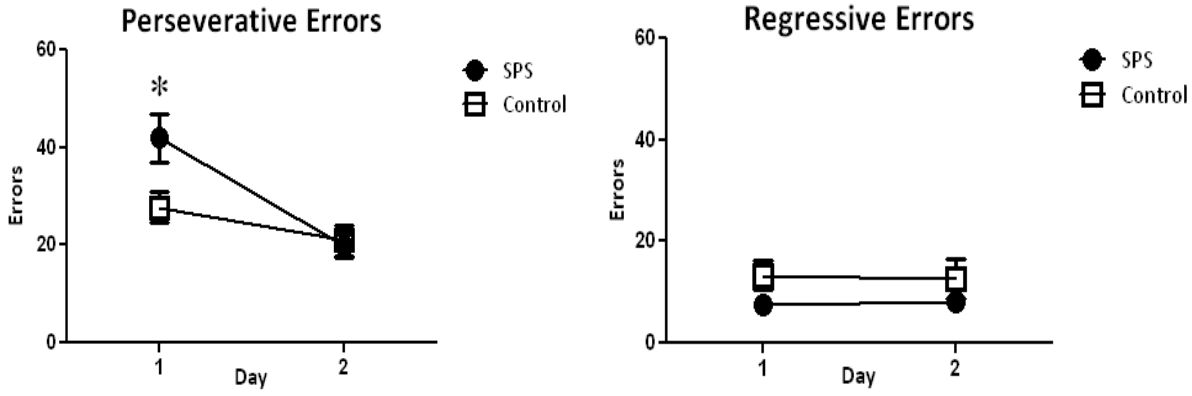

Figure 16. During reversal, SPS rats made more 'perseverative' errors on the first reversal but there was no difference in regressive errors.

Chronic antikindling drug administration attenuates SPS-induced deficits in extinction recall

In this experiment we evaluated the effect of SPS on extinction recall outside of the extinction context and determined if chronic administration of the anti-kindling drug Phenytoin can reverse these effects. This experiment was proposed in specific aim #4. Animals were subjected to SPS or control procedures, and then administration of the anti-kindling agent, Phenytoin, was repeated once per day for seven days. Phenytoin which prevents excitatory neural transmission by blocking voltage-gated sodium channels was found to prevent SPS-induced extinction retention deficits [$F(1,20)=5.73$, $p=.027$].

SPS sensitizes LC activity

We hypothesized that increased excitability of mPFC/amygdala activity in SPS rats is due to enhanced brain noradrenergic activity. In order to pursue this hypothesis and further address the aims of specific aim #4, we investigated the effects of SPS on single unit activity in the locus coeruleus (LC), the primary source of norepinephrine in the forebrain. SPS and control rats were subjected to stereotaxic surgery and single unit activity was recorded from the LC of rats under general anesthesia. SPS attenuated baseline single unit LC activity, but enhanced evoked single unit activity (induced by pinching the paw of rats) in the LC. This is shown in Figure 17. In another group of SPS and control rats we investigated whether SPS altered stress-induced upregulation of tyrosine hydroxylase (TH) mRNA in the LC. TH is the rate limiting enzyme in the norepinephrine biochemical cascade and changes in TH activity are correlated with the electrophysiological response of LC neurons. SPS and control were restrained for 1.5 hours in order to investigate the effect of SPS on TH mRNA upregulation. Data from 92 neurons (SPS = 50, Control = 42) were used in the analyses. SPS rats exhibited significantly lower baseline levels of spontaneous LC activity than controls [$t(90)=2.09$, $p=.039$] (Figure 17a). Quantification of LC discharge during components of the PSTH revealed that evoked LC activity of SPS rats was significantly higher than that of control rats [$t(90)=2.79$, $p=.006$] (Figure 17b). In contrast, tonic LC rate during the trial of noxious stimulation was not significantly different between

groups [t(90)=1.53, p=.13] (Figure 17c). The greater evoked discharge rate in the absence of a change in tonic rate resulted in a significantly higher signal-to-noise ratio in SPS compared to control rats [t(90)=3.02, p<.003] (Figure 17d). Between-group differences in peak LC response, latency to peak, and evoked response duration were also analyzed. Peak response tended to be higher in SPS animals (p<0.1), but no other differences were found between SPS and control animals for any of these LC discharge characteristics. Components of the histograms containing the evoked response were isolated and compared using repeated measures ANOVAs. The excitatory component of the PSTH was exaggerated in SPS rats (time x stress interaction [F(18,1620)=2.04, p=.025]). Figure 18a shows a representative autoradiogram of TH mRNA in the LC. Restraint stress increased TH mRNA expression in the LC of all animals [F(1,27)=30.05, p<.0001]. A two factor ANOVA revealed a significant interaction [F(1,27)=4.63, p=.040] indicating that SPS treatment differentially affected TH mRNA response to restraint stress (Figure 18b). Post-hoc comparisons indicated that baseline levels of TH mRNA expression tended to be lower in SPS rats than controls at trend level significance (t(9.71)=1.89, p=.089). To examine "reactivity" of the system controlling for baseline TH mRNA levels, we calculated TH mRNA expression as percentage increase from mean baseline scores and compared SPS and control groups. SPS rats demonstrated significantly greater increases in TH mRNA expression following restraint stress than controls [t(14)=3.21, p=.006] (Figure 18c). A manuscript detailing these findings has been accepted for publication in European Journal of Neuroscience, and is currently in press (George *et al.*, 2012).

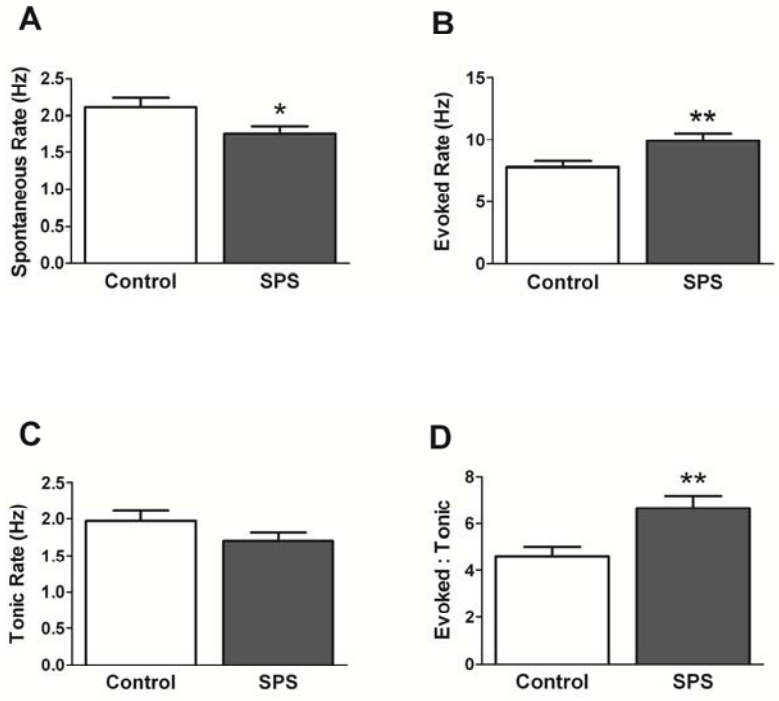

Figure 17. SPS effects on LC neuron discharge characteristics

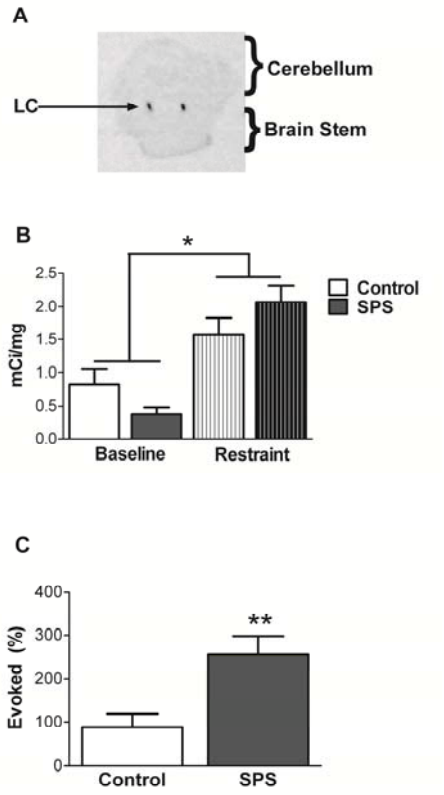

Figure 18. SPS effects on stress-induced TH mRNA expression in LC.

Key Research Accomplishments

- SPS animals demonstrate disruptions in the retention of extinction memories
- SPS augments the expression of GR in the prefrontal cortex and hippocampus, and this is linked to SPS-induced extinction retention deficits.
- SPS animals have decreased levels of glutamate in the mPFC suggestive of decreased excitatory neurotransmission in a brain region critical for emotion regulation.
- Some SPS effects can be attenuated by increasing mother-pup social interactions
- Inactivation of the BLA attenuates social interactions
- SPS impairs cognitive flexibility
- Administration of anti-kindling agent, Phenytoin attenuates extinction recall deficit
- SPS animals have enhanced noradrenergic reactivity in SPS as measured by single cell activity changes in the locus coeruleus and tyrosine hydroxylase mRNA levels.

Reportable Outcomes

The research we have conducted during the funded period has resulted in six peer reviewed journal publication, one article currently under review, and twelve conference abstracts. These are listed below.

Peer reviewed publications

George, SA., Knox, D., Curtis, A., Aldridge, JW., Valentino, RJ., Liberzon, I. Altered noradrenergic activity following Single Pronged Stress (in press, European Journal of Neuroscience).

Knox, D., Nault, T., Henderson, C., Liberzon, I. (2012a) Glucocorticoid receptors and extinction retention deficits in single prolonged stress model. Neuroscience. Oct 25;223:163-73.

Knox, D., Fitzpatrick, CJ., George, SA., Abelson, JL., Liberzon, I. (2012b) Unconditioned freezing is enhanced in an appetitive context: implications for the contextual dependency of unconditioned fear. Neurobiology of Learning and Memory. 97(4): 386-92

Knox, D., George, SA., Fitzpatrick, CJ., Rabinak, CA., Maren, S., Liberzon, I. (2012c) Single Prolonged Stress disrupts retention of extinguished fear in rats. Learning and Memory 19(2):43-9

Fitzpatrick, C., Knox, D., Liberzon, I. (2011). Inactivation of the prelimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces. Behavioural Brain Research: 22(1): 320 - 323. PMID:21420435

Knox, D., Perrine, S., George, SA., Galloway, M., Liberzon, I. (2010) Single Prolonged Stress decreases glutamate, glutamine and creatine concentrations in the rat medial prefrontal cortex. Neuroscience Letters 480, 16-20

Under review

Knox, D., Stout, S., Tan, M., George, SA., Liberzon, I. Early handling attenuates single prolonged stress enhancement of glucocorticoid receptor expression in prefrontal brain regions, but not hippocampus (under review, Neuroscience Letters).

Conference abstracts

George, SA., Riley J., Floresco, SB., Liberzon, I. Impaired cognitive flexibility following single prolonged stress, an animal model of PTSD. Society for Biological Psychiatry Annual Meeting, 2012. Philadelphia, PA.

George, SA., Knox, D., Curtis, AL., Valentino, RJ., Liberzon, I. Altered Locus Coeruleus activity following single prolonged stress, a rodent model of PTSD. American College of Neuropsychopharmacology Annual Meeting 2011. Kailua Kona, HI.

George, SA., Riley, J., Rodriguez, E., Floresco, S.B., Liberzon, I. Effects of single prolonged stress on cognitive flexibility. Society for Neuroscience Annual Meeting, 2011. Washington D.C., MD.

Fitzpatrick, CJ., Knox, D., George, SA., Liberzon, I. Neural mechanism by which single prolonged stress induces extinction deficits. Society for Neuroscience Annual Meeting, 2011. Washington D.C., MD.

Knox, D., Nault, T., Henderson, C., and Liberzon, I. Linking single prolonged stress-induced extinction deficits to single prolonged stress enhanced glucocorticoid receptor expression in limbic regions. Society for Neuroscience Annual Meeting, 2011, Washington D.C. MD.

George, SA., Knox, D., Curtis, A., Valentino, RJ., Liberzon, I. Altered noradrenergic activity following Single Pronged Stress, a rodent model of PTSD. Society for Biological Psychiatry Annual Meeting, 2011, San Francisco, CA.

George, SA., Knox, D., Fitzpatrick, CJ., Abelson, JL., Liberzon., I. Chronic Phenytoin treatment reverses stress enhanced renewal, but not reinstatement, of conditioned fear in an animal model of post-traumatic stress disorder. American College of Neuropsychopharmacology Annual Meeting, 2010, Miami, FL.

Stout S, Tan M, George S, Knox D, Stern ER, Liberzon I. The effects of early life and adult stress on HPA-axis function and anxiety-like behavior. Society for Neuroscience Annual Meeting, 2010, San Diego, CA.

Knox, D., and Liberzon, I. A comparison of the effects of TMT exposure and restraint stress on HPA axis function and noradrenergic systems. Society for Neuroscience Annual Meeting, 2010, San Diego, CA.

George, SA., Knox, D., Fitzpatrick, CJ., Maren, S., Abelson, JL. Liberzon, I. The effect of Single Prolonged Stress, a rodent model of PTSD, on extinction recall and reinstatement. Biological Psychiatry Annual Meeting, 2010, New Orleans, LA.

George, SA., Knox, D., Khan, S., Maren, S., Liberzon, I. The effect of Single Prolonged Stress, a rodent model of PTSD, on fear conditioning, extinction and extinction recall. Anxiety Disorders of America Association 2010, Baltimore, MD.

Knox, D., George, SA., Khan, S., Maren, S., Liberzon, I. The effect of Single Prolonged Stress, a rodent model of PTSD, on unconditioned anxiety, fear conditioning, extinction and extinction recall. American College of Neuropsychopharmacology Annual Meeting, 2009, Hollywood, Fl.

Conclusions

PTSD is a chronic, debilitating disorder that can emerge following exposure to a traumatic event. It is the 4th most common psychiatric disorder, with lifetime prevalence in the US at 6.8% (Kessler *et al.*, 2005). PTSD is characterized by a wide range of symptoms including hyperarousal, avoidance, intrusive memories and abnormalities in fear responses (American Psychiatric Association, 1994). Valid animal models are critical for understanding the neurobiological processes underlying psychopathology. Over the past decade our laboratory has developed a validated animal model of PTSD, Single Prolonged Stress (SPS) (Liberzon *et al.*, 1997) which produces multiple changes in physiology and behavior that resemble PTSD. Behaviorally, SPS animals exhibit enhanced acoustic startle (Khan & Liberzon, 2004), and neurobiologically, SPS reproduces the neuroendocrinological hallmark of PTSD (Yehuda *et al.*, 1993), enhancing fast negative feedback of the HPA axis (Liberzon *et al.*, 1999). The data collected during this funding period have demonstrated that the effects of SPS are not limited to HPA abnormalities. SPS animals have disruptions in the retention of extinction memories (Knox *et al.*, 2012b), a specific deficit exhibited by PTSD patients (Milad *et al.*, 2008). Failure to retain memories of safety contingencies has been proposed as a key psychobiological mechanism for the persistent fear and anxiety experienced by PTSD patients. We have also demonstrated that SPS augments the expression of GR in the prefrontal cortex and hippocampus, and that this is linked to SPS-induced extinction retention deficits (Knox *et al.*, 2012c), linking hallmark HPA abnormalities and specific behavioral deficits both implicated in PTSD. In addition, SPS animals have decreased levels of glutamate in the mPFC (Knox *et al.*, 2010), suggestive of decreased excitatory neurotransmission in a brain region critical for emotion regulation (Etkin & Wager, 2007). SPS neurobiological changes are not limited to these regions, as we have also demonstrated enhanced noradrenergic reactivity in SPS as measured by single cell activity changes in the locus coeruleus and tyrosine hydroxylase mRNA levels (George *et al.*, 2012). These findings further unravel the specific mechanisms that could link PTSD pathophysiology to specific symptom generation i.e. hyperarousal, extinction deficits etc. As we did not replicate some of the preliminary findings relating to social interaction and defense behavior regulation we have employed different paradigms to test these concepts. Following this line of research we have demonstrated that some of the effects of SPS can be attenuated by increasing mother-pup social interactions, that inactivation of the BLA attenuates social interactions, and that SPS impairs some forms of cognitive flexibility. We have also demonstrated that the prelimbic cortex plays a key role in inhibition of unconditioned fear, further supported by our findings that TMT-induced freezing (unconditioned fear) was enhanced in the appetitive context and that this enhancement was accompanied by decreased neural activity in the PL and IL (Knox *et al.*,

2012a). Finally we have collected preliminary data to suggest that administration of anti-kindling agents attenuates extinction recall deficits. Using the SPS model we have made significant advances in understanding neurobiological responses to trauma, demonstrating specific behavioral changes (PTSD symptoms) and changes in HPA axis, glutamatergic transmission and noradrenergic system activity. These findings have filled a considerable gap in our scientific understanding of the biological basis of PTSD, and provide the necessary foundation for future research into the development of novel strategies aimed at addressing the treatment and prevention of the full range of PTSD symptomatology.

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Knox, D., George, S.A., Fitzpatrick, C.J., Rabinak, C.A., Maren, S. & Liberzon, I. (2012b) Single prolonged stress disrupts retention of extinguished fear in rats. *Learn Mem*, **19**, 43-49.

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Appendix I

Statement of work

Institution name: University of Michigan Medical School, 1500 Medical Center Dr., Ann Arbor, MI 48109; Ann Arbor VA Healthcare Systems, 2215 Fuller Road, Ann Arbor, MI 48105.

Personnel and effort: Israel Liberzon MD, principal investigator (1.00 cal. months): Will oversee the whole project to ensure that all experiments are conducted in a timely fashion, consistent with the proposal, and in accordance with institutional guidelines and the principles of ethical use of animals in research. He will assure that all experiments are conducted with appropriate experimental rigor, and that data is published after completion of experiments. Dr. Liberzon will have primary responsibility for the interpretation of data and will primarily be responsible for writing research documents generated by this research proposal. Samir Khan PhD, co-investigator (6.00 cal. months): Will perform the bulk of experiments, data analysis, and research document preparation. Dayan Knox PhD, co-investigator (6.00 cal. months): Will perform the bulk of the experiments, data analysis, and research document preparation. Tony King PhD, co-investigator (0.60 cal. months): Will assist in developing protocols for protein and mRNA assays. Wayne Aldridge PhD, co-investigator (0.24 cal. months): will assist with electrophysiological experiments. TBA, research assistant (.60 cal. months years 1 & 2 and 6.00 cal. months years 3 & 4): will assist Drs. Khan and Knox in conducting all experiments. Stephen Maren PhD, consultant ($500 per year): will assist in electrophysiological and fear conditioning experiments.

General Tasks (6/1/08 – 6/31/08): 1) All equipment will be purchased within the first month of the release of funds to the University of Michigan. 2) All behavioral equipment will be purchased within the first month of the release of funds. 3) Electrophysiology equipment and molecular biology equipment required for testing hypotheses in specific aim 1 will be purchased within the first month of the release of funds to the University of Michigan.

Experimental Animals: Male Sprague Dawley rats will be used as subjects in all experiments. We anticipate an average of 15 rats/independent sample for all experiments in order to obtain statistical significance. However, this number will be adjusted from experiment to experiment used to test each hypothesis based on the difficulty of the experiments proposed, and the need for extra rats in the event that we are required to adjust the methods to deal with potential problems that may arise. In total we request 1,466 rats to complete the research proposal.

Animal protocol: All experiments will be staggered and a single animal protocol that includes all proposed experiments will be written in order to facilitate smooth transition from experiment to experiment. This protocol
will be written and submitted to the Veteran Affairs Institutional Animal Care Usage Committee within a month of notification that the University of Michigan has received the Intramural research award.

Proposed experiments: All experiments will be conducted between 6/1/08 – 5/31/12. Below we detail the experiments that we propose to conduct as they relate to each specific aim, and give time lines for the completion of these experiments. For all specific aims, the following sequence of tasks will be adhered to in order to allow for the execution of proper experimental protocols. A combination of temporary inactivation, single unit electrophysiology, pharmacological intervention, and molecular biology techniques will be used to test the hypotheses.

Tasks:

1) Purchase supplies for experiment (e.g. cannulas, electrodes, infusers, antibodies)
2) Purchase animals, perform SPS and/or drug procedures, and/or surgical procedures
3) Perform behavioral protocols (e.g. fear conditioning, extinction, social interaction)
4) Sacrifice rats and prepare tissue for histology or assay (e.g. Nissl stain, Western blot)
5) Perform assay (e.g. mRNA, protein), histology, or complete electrophysiology data analysis (within two weeks of termination of a particular experiment)
6) Repeat steps 2-5 at least once in order to replicate findings.

Specific Aim 1): ***Examine the roles of altered mPFC function and expression of brain glucocorticoid receptors in the development of SPS induced extinction deficits (as a model of PTSD intrusive symptom cluster).***	**No. of Animals**	**dates**
Hypothesis #1a: Temporary inactivation of the IL will lead to deficits in fear extinction in control rats, and this effect will be attenuated in SPS exposed rats. Methods used – cannula infusion, single prolonged stress, and fear conditioning	75	6/1/11 – 10/30/11
Hypothesis #1b: SPS exposure induces extinction deficits by altering neural activity in the IL. Methods used - Single unit electrophysiology, single prolonged stress and fear conditioning	45	1/11/11 – 5/31/11 (partially completed)
Hypothesis 1c: SPS exposure induces extinction deficits by altering brain glucocorticoid receptor expression. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	8/1/10 – 5/31/11 (completed)

Specific Aim 2): ***Examine the roles of altered mPFC/amygdala function in social interactions (as a model of PTSD social avoidance cluster), determine if social interactions can modulate SPS-induced changes in fear behaviors and HPA axis responses, and determine the importance of mPFC/amygdala activity and HPA/glucocorticoid receptor function in these SPS effects.***	**Number of Animals**	**dates**
Hypothesis #2a: Temporary inactivation of the IL will lead to avoidance of social interactions. Methods used – cannula infusion, in situ hybridization, and social	75	6/15/11 – 8/31/11

interaction test.		
Hypothesis #2b: Temporary inactivation of the BLA will increase social interactions. Methods used – cannula infusion, in situ hybridization, and social interaction test.	75	6/15/11 – 9/30/11 (completed)
Hypothesis #2c: Social buffering will not attenuate SPS-induced changes fear and stress reactivity. Methods used - Single prolonged stress, brief maternal separation, western blot electrophoresis, startle reactivity, fear conditioning	45	12/1/09 – 11/30/11 (completed)
Hypothesis #2d: Resistance to the beneficial effects of social buffering in SPS rats are due to aberrant neural activity in mPFC/amygdala circuits, and SPS-induced changes in the HPA axis. Methods used - Single prolonged stress, western blot electrophoresis, fear conditioning, in situ hybridization	90	10/1/10 – 8/31/11 (partially completed)

Specific Aim 3): ***Examine the role of altered mPFC/amygdala function and of altered HPA/glucocorticoid function in TMT-induced freezing, and determine if SPS disrupts social buffering of TMT-induced responses (as a model of emotional dysfunction in PTSD).***	**Number of Animals**	**dates**
Hypothesis #3a: Temporary inactivation of the IL will lead to deficits in defense behavior regulation similar to that observed in SPS animals. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	8/1/10 – 10/31/10 (completed)
Hypothesis #3b: Temporary inactivation of the BLA will attenuate the defense behavior regulation deficit induced by SPS. Methods used – cannula infusion, single prolonged stress, and predator induced freezing	75	11/1/10 – 01/31/11
Hypothesis #3c: SPS exposure induces deficits in regulation of defensive behavior by altering neural activity in the IL/BLA. C-fos in situ hybridization, single prolonged stress and predator induced freezing	45	9/1/11- 12/15/11 (completed)
Hypothesis #3d: SPS induced changes in defense behavior regulation are mediated, in part, by altered HPA axis/glucocorticoid function. Methods used - Western Blotting, in situ hybridization, reverse transcriptase polymerase chain reaction, and single prolonged stress.	90	5/1/11- 7/31/11

Specific Aim 4): ***Examine the ability of SSRI and antikindling drug administration to alleviate SPS induced extinction deficit and social buffering deficit; and the role of mPFC/amygdala activity, and***	**Number of Animals**	**dates**

HPA/glucocorticoid function in these processes.		
Hypothesis #4a. SSRI administration will attenuate SPS-induced extinction deficits and social buffering deficits by altering IL/BLA electrophysiological activity in SPS animals and by reversing changes in glucocorticoid receptor and mRNA expression in the prefrontal cortex, hippocampus, and hypothalamus. Methods used - Single unit electrophysiology, single prolonged stress, fear conditioning, social interaction, predator induced freezing, western blotting, in situ hybridization, and reverse transcriptase polymerase chain reaction.	359	8/1/11 – 1/31/12
Hypothesis# 4b. Antikindling/mood stabilizer administration will attenuate SPS induced defensive behavior regulation deficits by modulating neural activity in the IL/BLA. (two different drugs will be tested). Methods used - Single unit electrophysiology, single prolonged stress, and predator induced freezing.	327	2/1/12 – 5/31/12 (partially completed)

Appendix II

Individuals paid on this grant are as follows: Israel Liberzon, Dayan Knox, Sophie George, Anthony King, Wayne Aldridge, Christopher Fitzpatrick, Hedieh Briggs, Nirmala Rajaram, Lan Xiao.

Appendix III
Original manuscripts

Behavioural Brain Research 221 (2011) 320–323

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Short communication

Inactivation of the prelimbic cortex enhances freezing induced by trimethylthiazoline, a component of fox feces

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ABSTRACT

Previous research has demonstrated that the rodent medial prefrontal cortex (mPFC) is critical for the expression of unconditioned defense behaviors. The prelimbic (PL) and infralimbic (IL) cortices comprise the majority of the mPFC, but the role of these regions in mediating unconditioned defense behaviors is not well understood. In order to address this, we temporarily inactivated the PL or IL and documented the effects of these manipulations on freezing induced by trimethylthiazoline (TMT), a component of fox feces, and center region avoidance in the open field (OF). PL inactivation enhanced TMT-induced freezing, but had no effect on OF behavior. IL inactivation had no effect on any behavioral measure. The results of this study are the first to demonstrate that the PL can have an inhibitory role with regard to unconditioned defense behavior. Further research is needed to define the parameters under which the PL inhibits unconditioned defense behavior.

Previous research has demonstrated that the rodent medial prefrontal cortex (mPFC) is critical for mediating unconditioned defense behaviors. Permanent lesions of the mPFC, restricted to the prelimbic (PL) and infralimbic (IL) cortices, attenuate anxiety-like behavior in the open field (OF) [1], elevated plus maze (EPM) [1,2], social interaction [3], and shock-probe burying [3] tests. Similar effects are observed with temporary inactivation [4].

Given that the PL and IL are differentially interconnected [5,6] and potentially exert differential effects on behavior [7], the differential roles of these sub-regions in mediating unconditioned defense behavior need to be examined. Previous studies have examined this relationship, but the roles of the PL and IL in unconditioned defense behavior are still not well understood. First, with respect to the PL, the existing findings are somewhat confusing. PL inactivation increases open arm behavior in the EPM (an anxiolytic effect) in one study [8], but has no effect on center region avoidance in the OF or freezing induced by a predator in another study [9]. Second, temporary inactivation of the PL or IL increases punished licking in the Vogel conflict test [10], which is quite surprising given that the PL and IL have different connectivity patterns [5,6]. Previous studies have examined the roles of the PL and/or IL in mediating an isolated defense behavior [10] or the role of the PL in mediating a number of defense behaviors [9]. Given the differential connectivity patterns of these mPFC regions and the likelihood that unconditioned defense behaviors are not supported by identical neurocircuitry, it is prudent to examine the role of the PL and IL in mediating different defense behaviors in the same study. Till date, this has not been done. Thus, while the results of previous studies suggest that the mPFC is critical for expressing unconditioned defense behaviors, the specific roles of the PL and IL in mediating these behaviors require further investigation.

To address this, we temporarily and selectively inactivated the PL or IL and observed the effects of these manipulations on two unconditioned defense behaviors: freezing induced by trimethylthiazoline (TMT), a component of fox feces, and OF behavior. We used the TMT-induced freezing paradigm, because TMT selectively enhances multiple defense behaviors when compared to other aversive odors such as butyric acid [11–14], supports contextual fear conditioning [15], and activates brain regions that are critical for the expression of unconditioned defense behaviors, including the PL and IL [16,17]. The OF test was selected because it is commonly used and widely accepted test of anxiety [18].

Thirty-six male Sprague Dawley rats (Charles River, Wilmington, MA) weighing 210–270 g at the time of surgery were used in this study. The animals were maintained on a 12 h light/dark cycle in a room maintained at 19–21 °C and 50 ± 10%

Abbreviations: BLA, basolateral complex of the amygdala; EPM, elevated plus maze; IL, infralimbic cortex; OF, open field; mPFC, medial prefrontal cortex; PAG, periaqueductal gray; PL, prelimbic cortex; PSB, pontamine sky blue; T1–4, time period 1–4; TMT, trimethylthiazoline.

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humidity. Rats had *ad libitum* access to food and water and were allowed to acclimatize to the colony room for three days prior to surgery. All experimental procedures were approved by the local animal care committee (Veterans Affairs IACUC, Ann Arbor, MI) and in accordance with National Institutes of Health guidelines for the treatment of animals.

Prior to general anesthesia, rats were administered the muscle relaxant xylazine (Anased®, Ben Venue Laboratories, Bedford, OH; 20 mg/kg) subcutaneously (s.c.). General anesthesia was induced with 5% isoflurane (Flurane®, Baxter Healthcare Corporation, Deerfield, IL) in oxygen flow, and maintained at 1–2% isoflurane in oxygen flow during surgery. Depth of anesthesia was measured by the limb withdrawal and eye blink reflexes. No surgical procedures were conducted until both reflexes were absent. To prevent anesthesia-induced hypothermia, rats were warmed with a water-circulating heat pad (Gaymar Industries, Orchard Park, NY).

Stereotaxic surgery was performed by securing rats in a Kopf stereotaxic frame (Tujunga, CA), and rats were bilaterally implanted at 10°, to the *y*-axis, with stainless steel cannulae (26-gauge, 10 mm; Plastics One, Roanoke, VA). The stereotaxic coordinates for cannulae implantation were as follows: for PL target (*A*: +2.7 mm, *L*: ±0.4 mm, *D*: −2.4 mm) and for IL target (*A*: +2.7 mm, *L*: ±0.4 mm, *D*: −3.6 mm). All coordinates were referenced from bregma and based on the atlas of Paxinos and Watson [19]. Cannulae and three bone screws were bonded to the skull with dental acrylic. Dummy cannulae were inserted into the guide cannulae to maintain their viability. After surgery, rats were administered 3 mL of sterile saline s.c. to prevent dehydration. Experimentation continued when rats regained their pre-surgical body weight followed by two days of consecutive weight gain.

The PL and IL were temporarily inactivated by infusion of the sodium channel blocker, lidocaine HCL (Sigma–Aldrich, St. Louis, MO) in a concentration of 2%. Lidocaine HCl was dissolved in a 0.9% saline solution, which was also used as the vehicle treatment. A microsyringe pump controller (Harvard Apparatus, Holliston, MA) with 5 μL syringes (Hamilton Company, Reno, NV) was used for infusions. Polyethylene tubing connected infusion cannulae (33-gauge, 11 mm; Plastics One, Roanoke, VA) to the syringes and controller. Solutions were infused bilaterally at a rate of 0.2 μL/min for 1 min [10] and infusion cannulae remained in the guides for an additional minute. Behavioral procedures commenced 15 min later.

To test the role of the PL and IL in mediating TMT-induced freezing, the PL or IL of rats was temporarily inactivated 15 min prior to placement in a test chamber that contained TMT. The test chamber was 36 cm width × 34.5 cm length × 52 cm height, illuminated by red light, and contained a piece of filter paper in one corner that had 15 μL of TMT in neat form. Behavior was recorded for 8 min by a camera mounted on a wall of the test chamber and scored at a later date. After each experiment, the chambers were wiped clean with 70% ethanol and aerated.

To test the effect of PL and IL inactivation on anxiety-like behavior in the open field (OF), the PL or IL of rats was temporarily inactivated 15 min prior to placement in the OF (91.5 cm width × 91.5 cm length × 61 cm height), which was illuminated with red light. A grid was drawn on the floor of the arena dividing it into 25 segments (19.02 cm width × 19.02 cm length). In addition, the arena was divided into a periphery (15.25 cm from the walls) and center (61 cm × 61 cm). Rats were placed in the center of the OF and activity was recorded by a camera for 5 min, then analyzed at a later date. Lidocaine and saline infusions were performed on separate groups of rats. The order of TMT-induced freezing and OF testing was counterbalanced for all rats and each behavioral test was separated by 24 h. If data for a behavioral test from a rat was lost (e.g. loss of OF data due to camera malfunction), then the exact experimental procedure was repeated in another rat, but only for the lost behavioral test.

Upon completion of the final experiment, rats were infused with a 2% pontamine sky blue solution (PSB) through the guide cannulae, decapitated, and their brains were removed and frozen in isopentane over dry ice. Coronal sections were then cut at 30 μm within a cryostat (Leica Microsystems Inc., Bannockbum, IL), and then stained for Nissl substance. These procedures were adopted to mark the location of infuser tips within the brain.

In order to verify correct cannulae placements, locations of PSB infusions were mapped onto standardized coronal sections of a rat brain stereotaxic atlas [19]. Only rats with correct cannulae placements were considered for final analysis. For the TMT experiment, freezing (defined as the absence of movement, with the exception of respiration, for greater than 3 s) was scored by an individual unaware of the experimental groups to which the rats were assigned. Freezing was analyzed in 2 min periods over an 8 min exposure trial, to yield four time periods (T1–4). Freezing scores during these time periods were subjected to a mixed measure two factors design with the independent factor being drug (vehicle vs. lidocaine) and the repeated measure being time (T1–4). This factor design was used to separately analyze the effect of PL and IL inactivation on TMT-induced freezing. Main and simple effects were analyzed using analysis of variance, while main and simple comparisons were analyzed using *t*-test with a Bonferroni correction applied.

For the OF test, center region avoidance was measured by documenting time spent in, and entries made into, the center of the field, while segment crossings was used a measure of locomotor activity (i.e. non-defense behavior). A segment crossing was defined when more than three quarters of a rat's body entered into a distinct segment. All behaviors were analyzed using *t*-test (drug: saline vs. lidocaine). *t*-Test was used to separately analyze the effect of PL or IL inactivation on OF behavioral measures. The criterion for statistical significance was set to $p < 0.05$.

Fig. 1 illustrates representative microphotographs, taken at 1.5× with a stereoscopic zoom microscope (Nikon, Melville NY), of bilateral infusion cannulae placements in the PL and IL, and schematics of all correct PL and IL placements in this study. Only data from rats with correct PL (vehicle = 8, lidocaine = 8) and IL (vehicle = 12, lidocaine = 8) cannulae placements were used for statistical analysis.

Fig. 2A–D illustrates the effects of PL inactivation on defense behaviors documented in this study. PL inactivation enhanced TMT-induced freezing, [Fig. 2A, main effect of drug on TMT-induced freezing, [$F(1,14) = 21.954$; $p < .0001$]), but had no effect on time spent in the center of the OF [Fig. 2B, $t(12) = .722$; $p = 0.455$], center region entries [Fig. 2C, $t(12) = .022$; $p = 0.983$], or general locomotor activity reflected by segment crossings [Fig. 2D, $t(12) = 0.359$; $p = 0.726$]. Fig. 2E–H illustrates the effects of IL inactivation on defense behaviors documented in this study. IL inactivation had no effect on TMT-induced freezing [Fig. 2E, $F(1,14) = 0.154$; $p = 0.701$], center time in the OF [Fig. 2F, $t(12) = 0.803$; $p = 0.438$], center entries in the OF [Fig. 2G, $t(12) = 0.216$; $p = 0.833$], or segment crossings in the OF [Fig. 2H, $t(12) = 0.415$; $p = 0.685$].

The results of the current study demonstrate that PL inactivation selectively enhances TMT-induced freezing. This was not caused by non-specific effects of PL inactivation on locomotion, because PL inactivation did not affect locomotion in the OF, as indexed by number of segment crossings. Furthermore, PL inactivation had no effect on center region avoidance (i.e. defense behavior) in the OF. Given that presentation of TMT increases neural activity in the PL [16,17], the results of this study suggest that PL neural activity is critical for inhibiting TMT-induced freezing. These effects appear selective to the PL, because IL inactivation had no effect on comparable behavioral tests.

Previous work has demonstrated that the IL plays a key inhibitory role in conditioned freezing paradigms, such that increasing IL neural activity inhibits conditioned freezing [20] and

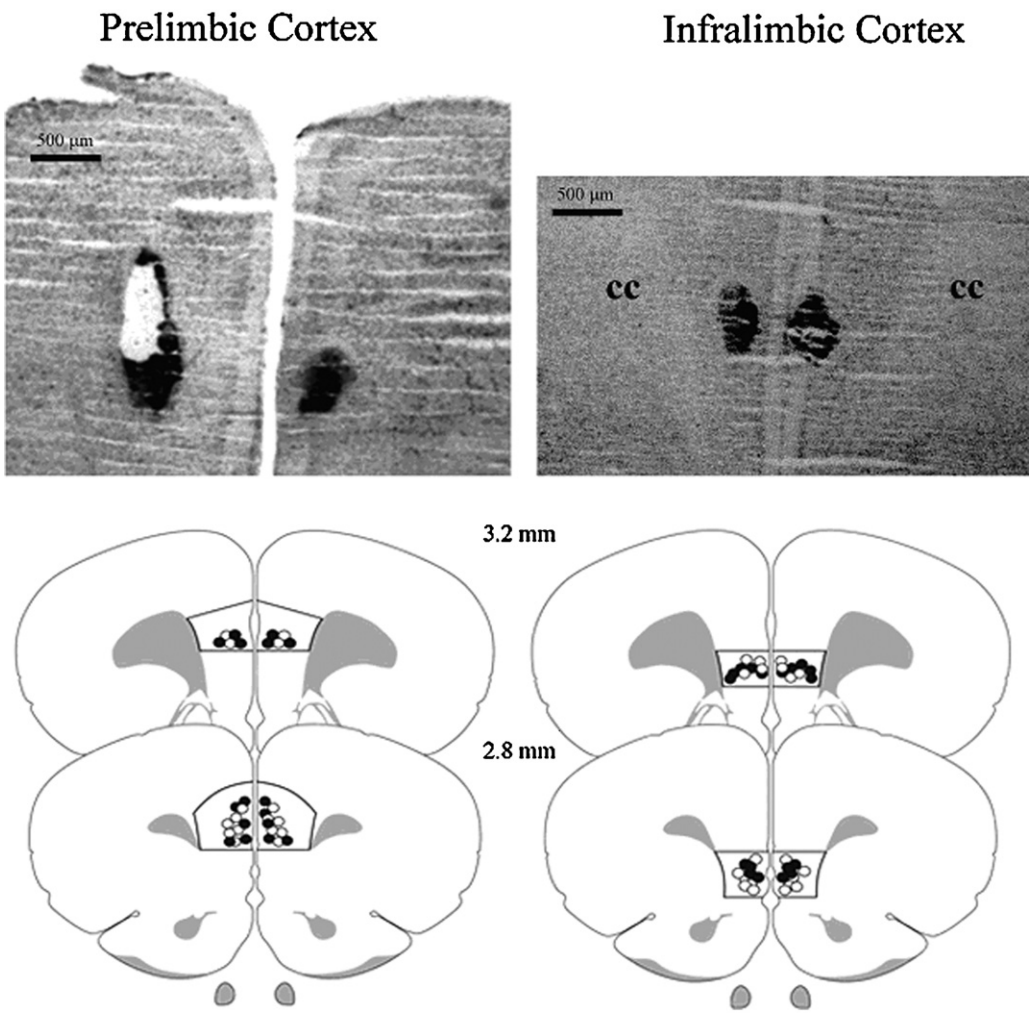

Fig. 1. Infusion cannulae placements in the prelimbic (PL) and infralimbic (IL) cortices. (top left panel) Microphotograph of bilateral infusion cannulae placements in the PL and (top right panel) IL. The tips of infusion cannulae are marked with pontamine sky blue. (bottom left panel) Schematic of all PL and (bottom right panel) IL infusion cannulae placements in this study. The numbers next to the schematics represent distance from Bregma. Only data from rats with correct bilateral PL and IL cannulae placements were used. cc—corpus callosum.

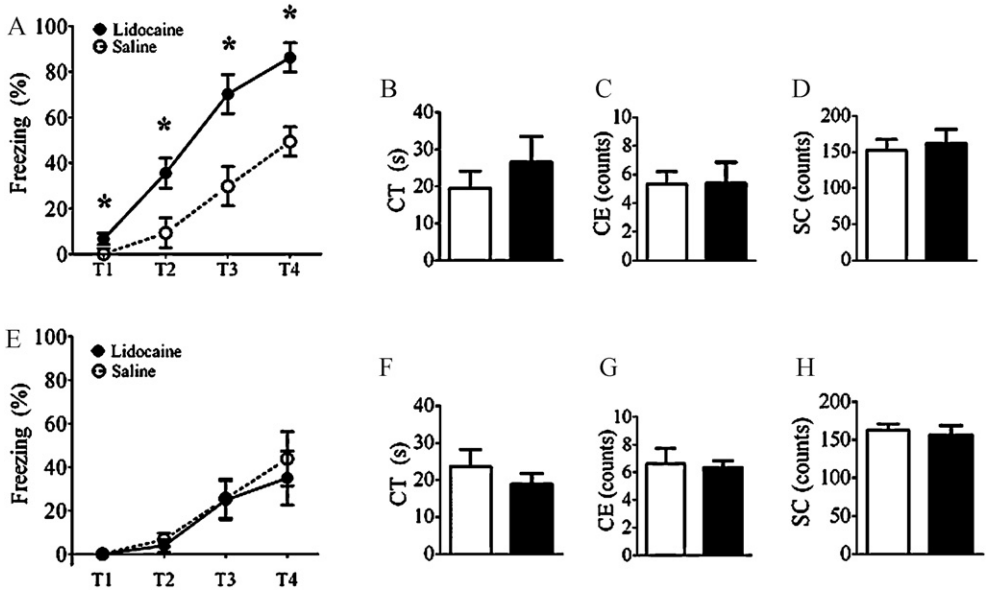

Fig. 2. Effect of PL and IL inactivation on predator odor-induced freezing and OF behavior. (A) PL inactivation enhanced TMT-induced freezing, but had no effect on (B) center region time, (C) center region entries, or (D) segment crossings in the OF. (E) IL inactivation had no effect on TMT-induced freezing, (F) center region time, (G) center region entries, or (H) segment crossings in the OF. TMT—trimethylthiazoline, OF—open field, CT—center time, CE—center entry, SC—segment crossing, *—significant main effect of drug.

inhibition of IL neural activity enhances conditioned freezing during fear re-extinction [21]. A similar key role for the mPFC with regard to unconditioned freezing has not been demonstrated until this study. IL inhibition of conditioned freezing may be accomplished by IL inhibition of neural activity in ventro-caudal brain regions [22,23] involved in expression of conditioned freezing, like amygdaloid nuclei. This suggests the PL might also inhibit unconditioned freezing by inhibiting neural activity in neural substrates involved in expression of unconditioned freezing. What might these anatomical substrates be?

The PL has a stronger innervation of the BLA and periaqueductal gray (PAG) when compared to the IL [5,6]. Temporary inactivation of the BLA inhibits the onset of TMT-induced freezing [24] and stimulation of the PAG induces freezing [25,26]. Thus, PL inactivation may enhance TMT-induced freezing by disinhibiting neural activity in the BLA and PAG. Further research is needed to test this hypothesis.

A previous study has reported that PL inactivation has no effect on freezing induced by predator presentation [9], which may appear contradictory to the results of this study. Presentation of predator cues signal regarding the potential presence of a predator, and can be considered a distal threat [27,28], whereas presentation of an actual predator represents imminent danger [28,29]. Thus, predator presentation may simultaneously activate several fear circuits in the brain (e.g. medial amygdala, central nucleus of the amygdala, BLA, bed nucleus of the stria terminalis, PAG) to such an extent that inhibitory modulation by the PL has no effect on freezing behavior. As a result, PL inactivation would have no effect on predator-induced freezing. Indeed, in Corcoran and Quirk [9], freezing levels were twice as high as those observed on our study. Other technical differences in testing apparatus (e.g. size of testing arena) and in protocol could also contribute to observed differences. Also, the finding that PL inactivation had no effect on OF behavior is not surprising, because the OF test does not involve unconditioned freezing.

Previous experiments have demonstrated that PL inactivation attenuates certain unconditioned defense behaviors [8,10]. The varied effects of PL inactivation on defense behavior suggest that neurocircuitry which mediates different unconditioned defense behaviors are not identical and that the role of the PL within these circuits is also different (e.g. inhibitory vs. facilitatory). Given that unconditioned defense behaviors are used to model fear and anxiety [27,28], further research investigating the role of the PL in mediating fear and anxiety is needed.

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Single Prolonged Stress Decreases Glutamate, Glutamine, and Creatine Concentrations In The Rat Medial Prefrontal Cortex

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Abstract

Application of Single Prolonged Stress (SPS) in rats induces changes in neuroendocrine function and arousal that are characteristic of Post Traumatic Stress Disorder (PTSD). PTSD, in humans, is associated with decreased neural activity in the prefrontal cortex, increased neural activity in the amygdala complex, and reduced neuronal integrity in the hippocampus. However, the extent to which SPS models these aspects of PTSD has not been established. In order to address this, we used high-resolution magic angle spinning proton magnetic resonance spectroscopy (HR-MAS ^{1}H MRS) *ex vivo* to assay levels of neurochemicals critical for energy metabolism (creatine and lactate), excitatory (glutamate and glutamine) and inhibitory (gamma amino butyric acid (GABA)) neurotransmission, and neuronal integrity (N-acetyl aspartate (NAA)) in the medial prefrontal cortex (mPFC), amygdala complex, and hippocampus of SPS and control rats. Glutamate, glutamine, and creatine levels were decreased in the mPFC of SPS rats when compared to controls, which suggests decreased excitatory tone in this region. SPS did not alter the neurochemical profiles of either the hippocampus or amygdala. These data suggest that SPS selectively attenuates excitatory tone, without a disruption of neuronal integrity, in the mPFC.

Keywords

PTSD; anxiety; emotional regulation; glutamate; GABA; proton magnetic resonance spectroscopy

Introduction

The Single Prolonged Stress (SPS) paradigm refers to the serial application of restraint stress, forced swim, and ether exposure, followed by a quiescent period of seven days. This paradigm has been developed as a rat model of Post Traumatic Stress Disorder (PTSD). PTSD patients show augmented fast negative feedback of the hypothalamic-pituitary-

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adrenal (HPA) axis [1] and augmented startle reactivity [2–5] and previous reports indicate that SPS models these aspects of PTSD [6,7]. Recently, a specific deficit in recall of extinguished fear conditioning has been reported in PTSD patients [8] and disrupted extinction recall has also been reported in animals that have undergone SPS treatment [9]. Taken together, these findings further support the validity of SPS in rats as a model of specific neuroendocrine and behavioral changes associated with PTSD.

Changes in neural function in the medial prefrontal cortex (mPFC), amygdala complex, and hippocampus are also characteristic of PTSD. When presented with reminders of traumatic events PTSD patients show attenuated hemodynamic responses in the ventromedial prefrontal cortex [10,11] and elevated responses in the amygdala complex [12,13] when compared to control subjects. These alterations in neurocircuitry relating to fear processing and emotion regulation have been proposed as mechanisms for pathological fear in PTSD, where decreased mPFC input to the amygdala fails to appropriately inhibit conditioned fear responses [for a recent review see 14]. Indeed, excitatory projections from glutamatergic neurons in the mPFC have been implicated in the inhibition of principle amygdala neuron firing in rats [15]. In addition, it has been consistently reported that PTSD patients demonstrate reduced hippocampal volume and decreased neuronal integrity (i.e. the destruction of neurons, decreased neuronal density, or aberrant changes in cellular processes within neurons) in the hippocampus [12,16,17] in comparison to controls. To date, the effects of SPS on neural activity in the mPFC and amygdala, and neuronal integrity in the hippocampus or hippocampal volume, have not been evaluated.

Magnetic resonance spectroscopy (MRS) can be used to assess the concentrations of multiple neurochemicals within the brain (*in vivo*) or in a single brain sample (*ex vivo*), allowing some inference regarding aspects of neural function in the brain. For example, N-acetylaspartate (NAA) is a neurochemical expressed in neurons and neuronal processes, but not glia [18,19]. NAA is indicative of neuronal death [19], decreases in neural density [19,20], and aberrant metabolic processes [20,21]. NAA is reduced in a number of disorders associated with decreased neuronal integrity. These include brain tumors [22], epilepsy [23], and multiple sclerosis [24]. Thus, by measuring levels of NAA one can infer levels of neuronal integrity in the brain [19,21].

While MRS cannot be used to measure neural activity (i.e. changes in membrane potential over a given period of time) the technique can be used to index the levels of neurochemicals critical to changes in neural activity. For example, by measuring relative concentrations of inhibitory (i.e. gamma amino butyric acid (GABA)) and excitatory (i.e. glutamate) neurotransmitters, and of molecules indicative of energy metabolism which are necessary for changes in membrane potential, the potential for increased neural activity can be inferred (i.e. excitatory tone). MRS technology has been applied to study the neural basis of psychiatric disorders. Changes in hippocampal neuronal integrity in PTSD patients have been reported, in proton (^{1}H) MRS studies, that measure NAA levels *in vivo* [25–28], suggesting that MRS technology can be useful to further investigate neural processes critical to the etiology of PTSD.

The goal of this study was to determine the effect of SPS on levels of excitatory and inhibitory neurotransmitters, neurochemicals indicative of energy metabolism, and NAA in the mPFC, amygdala, and hippocampus. In order to accomplish this, we used high-resolution magic angle (HR-MAS) ^{1}H MRS *ex vivo* methodology, studying mPFC, amygdala, and hippocampal tissue. Glutamate, GABA and glutamine (the major metabolite of neuronal glutamate [29–32]); chemicals that are components of biochemical pathways that result in ATP production (e.g. succinate for the Krebs cycle, lactate for glucose metabolism, and creatine for ATP production via creatine phosphate); and NAA levels

(indicative of neuronal integrity) were measured. We predicted that SPS would attenuate the levels of neurochemicals indicative of excitatory neurotransmission and energy metabolism in the mPFC (i.e. excitatory tone), augment the levels of chemicals indicative of excitatory neurotransmission and energy metabolism in the amygdala complex, and attenuate NAA levels (i.e. disrupt neuronal integrity) in the hippocampus.

Materials and Methods

Animals

Fifteen male Sprague Dawley (SPS = 8, control = 7) rats (Charles River, Wilmington, MA) were pair-housed at the Veterinary Medical Unit of the Ann Arbor Veterans Affairs medical center and maintained on a 12:12 hour light/dark cycle, 19 – 21°C room temperature, and 50 ± 10% humidity. The animals had *ad libitum* access to food and water. All experimental procedures were approved by the Ann Arbor Veteran Affairs Institutional Animal Care Usage Committee and were in accordance with the National Institute of Health Guide For The Care and Use of Laboratory Animals. Animals were allowed to acclimatize to the colony room for at least 3 days prior to the initiation of experiments.

Single Prolonged Stress

SPS refers to the application of three stressors (restraint stress, forced swim, and ether exposure) followed by a quiescent period of 7 days [33,34]. In this study, rats were restrained for 2 hours, followed immediately by 20 minutes of forced swimming in 20 – 24 °C water in a plastic tub (55.6 cm diameter, 45.4 cm height), filled two-thirds from the bottom. Following 15 minutes recuperation, rats were exposed to ether (using a dessicator) until general anesthesia, defined as loss of toe and tail pinch responses, was induced (< 5 minutes). Immediately after the induction of general anesthesia, rats were removed from the dessicator, placed in their home cages, and left undisturbed for 7 days. For the control procedure, rats remained in their home cages for the duration of SPS [34].

Brain Dissections

Previous studies have demonstrated that increases in fast negative feedback of the HPA axis and decreases in the ratio of MR/GR mRNA in the hippocampus are observed up to 14 days after SPS [33,34]. As a result rat brains were harvested within this time window. Three days after SPS (that is 10 days after the application of three stressors) all rats were decapitated without anesthesia; their brains rapidly removed, frozen on dry ice and stored at −80 °C until being transported to Wayne State University for neurochemical analysis. Whole frozen brains were packed in dry ice, delivered overnight, and once received were stored at −80 °C until further dissection to isolate specific brain regions. In order to prepare samples for HR-MAS ^{1}H-MRS analysis, brains were placed into an ice-cold matrix, allowed to thaw enough to cut with a razor blade, and then sliced into 2 mm coronal sections. Slices containing the mPFC (prelimbic and infralimbic regions), amygdala complex (basolateral complex and central nucleus), and hippocampus (CA1 dentate gyrus region) were obtained and then specific regions were microdissected using a punch technique (2.1 mm diameter). Sample-punches were frozen immediately on dry ice and stored at −80 °C until HR-MAS ^{1}H-MRS analysis.

HR-MAS ^{1}H-MRS

Neurochemical profiles were determined with HR-MAS ^{1}H-MRS as previously described [35–37]. Briefly, frozen intact tissue samples were weighed (~ 3 mg) and placed directly into a Bruker zirconium rotor (2.9-mm diameter, 10 μL capacity) containing 5 μL buffer (pH = 7.4; 100 mM potassium phosphate, 200 mM formate, 1 g/L NaN_3 and 3 mM

trimethylsilyl-propionate [TSP Sigma; St Louis, MO] diluted with an equal volume of D_2O containing 0.75% TSP). TSP serves as an internal chemical shift reference (0.00 ppm), formate (8.44 ppm) for phase corrections, and D_2O to lock on the center frequency. The rotor (with sample) was placed into a Bruker magic angle spinning probe maintained at 4 °C in a vertical wide-bore (8.9 cm) Bruker 11.7 T magnet with an AVANCE™ DRX-500 spectrometer (Bruker Biospin Corp., Billerica, MA). Rotors were spun at 4.2 ± 0.002 kHz while positioned at 54.7° relative to the static magnetic field B_0. A Carr–Purcell–Meiboom–Gill (CPMG) rotor-synchronized pulse sequence [38] (TR = 3500 ms, bandwidth 8 kHz, 16 k complex points, 32 averages) was used to acquire the spectra with a total acquisition time of 3 minutes 38 s. Spectra were analyzed with a customized Linear-Combination Model (LC Model) software package that uses a linear combination of 27 individual neurochemical model spectra (basis set) as well as non-specific lipid signals to fit the tissue spectrum, and calculates absolute concentration values for neurochemicals with signals between 1.0 – 4.2 ppm [39]. Cramer–Rao bounds estimated the precision with which LCModel fit the data and were typically below 10% indicating excellent fit. Absolute values were corrected for tissue sample weight and expressed as nmol/mg of wet weight. The statistical significance of differences observed between SPS and control was assessed for each neurochemical with a two-tailed Student's t-test (SPS vs. control) with a 95% confidence interval ($p < 0.05$).

Results

Figures 1A–C illustrates the metabolites used to index neuronal integrity and energy metabolism in the mPFC (SPS = 7, Control = 6), amygdala complex (SPS = 7, Control = 6), and hippocampus (SPS = 8, Control = 7). Of these metabolites, only creatine levels in the mPFC were attenuated by SPS [$t(11) = 2.63$, $p = .023$]. Glutamate [$t(11) = 2.912$, $p = .014$] and glutamine [$t(11) = 2.445$, $p = .033$] levels were also attenuated in the mPFC of SPS rats when compared to controls, while GABA levels were not different [$t(11) = 1.404$, $p = .18$]. These results are illustrated in Figure 2A. Neither glutamate, glutamine, nor GABA levels in the hippocampus [glutamate - $t(13) = 1.279$, $p = .223$; glutamine - $t(13) = 1.335$, $p = .205$; GABA $t(13) = .482$, $p = .638$] or amygdala complex [glutamate - $t(11) = .044$, $p = .966$; glutamine - $t(11) = .198$, $p = .846$; GABA - $t(11) = .620$, $p = .548$] were altered by SPS. These results are illustrated in Figures 2B–C.

Discussion

In this study SPS attenuated creatine, glutamate, and glutamine, but not NAA levels in the mPFC of the SPS rats. Glutamate is involved in metabolic processes that are not related to neural transmission, which raises the possibility that changes in glutamate observed in this study did not concern glutamate used for neural transmission in the mPFC. However, we observed attenuated glutamine, the precursor metabolite for neuronal glutamate [29,31,32,40], concentrations in the mPFC of SPS rats. In addition, glutamate serves as a precursor molecule for the synthesis of GABA [30,41,42], but GABA levels in the mPFC were not affected by SPS. Furthermore, succinate levels, which are indirectly dependent on glutamate levels [30], were not affected by SPS. This change in neurochemical profile is consistent with the assertion that SPS induces a decrease in excitatory tone in the mPFC, without a corresponding change in neuronal integrity, and provides evidence for a SPS-induced deficit in neural activity in the mPFC.

SPS did not alter NAA levels in the hippocampus. Given that decreased hippocampal NAA has been reported in PTSD [17,25,26,28], it appears that SPS does not model this aspect of PTSD. SPS did not alter neurochemical profiles in the amygdala, and amygdala hyperactivity has been linked to PTSD by our group and other investigators [12,13]. It should be noted, however, that while observed changes in inhibitory and excitatory

neurotransmitter levels and energy metabolites can be used to make inferences about excitatory tone in a brain region, the converse is not necessarily true. Thus, it is possible to have changes in neural activity that would not be detected by HR-MAS ^{1}H-MRS. Changes in the sensitivity of glutamate receptors, for instance, could alter the electrochemical gradient of positive ions such as sodium and calcium. Such changes could lead to increased membrane excitability in the amygdala without a detectable change (using HR-MAS ^{1}H-MRS technology) in neurochemical concentrations. It is also possible that the amygdala hyperactivity reported is a functional outcome of diminished prefrontal inhibitory tone, without intrinsic intra-amygdala neurobiological alterations. Thus, further research is needed to examine the effect of SPS on excitatory tone in the amygdala.

Decreased neural activity in prefrontal cortical regions, without a change in neuronal integrity, is believed to be a salient feature of PTSD [10,11]. The results of this study suggest that SPS attenuates excitatory tone in the mPFC without a change in neuronal integrity. This suggests that SPS can be used to model prefrontal cortical dysfunction associated with PTSD. Previous research has demonstrated that acute stress-induced changes in glutamate metabolism are caused by stress-induced increases in corticosterone concentrations [43,44]. SPS does not affect baseline (laboratory observation) or stress-induced increases in corticosterone concentrations [34], which demonstrates that corticosterone-induced changes in glutamate metabolism is not the mechanism by which SPS alters glutamate function in the mPFC. Further research is needed to determine the mechanism by which SPS selectively attenuates glutamate metabolism in the mPFC. The results of this study suggest that SPS decreases neural activity in the mPFC. This assertion is also supported by the finding that SPS induces deficits in extinction recall [50], because decreased neural activity in the mPFC disrupts extinction recall [36,44]. Further research is needed to investigate the effects of SPS on direct measures of neural activity (e.g. single unit activity) in the mPFC.

Acknowledgments

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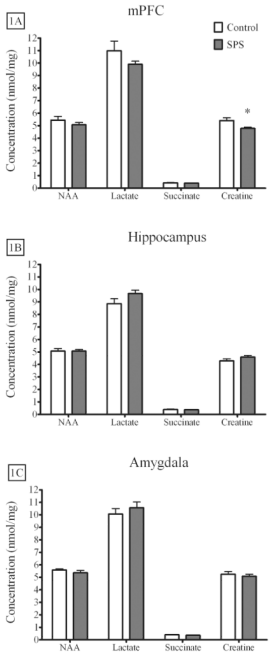

Figure 1.

The effect of SPS on energy metabolites and NAA in the mPFC, hippocampus, and amygdala complex. SPS attenuated creatine levels in the A) mPFC, but had no effect on neurochemical profiles in the B) hippocampus or C) amygdala complex. Data are expressed as nmol/mg tissue, presented as the mean ± standard error of the mean, and analyzed by two-tailed t-test (* $p < 0.05$). SPS – Single Prolonged Stress, NAA – N-acetyl aspartate, mPFC – medial prefrontal cortex.

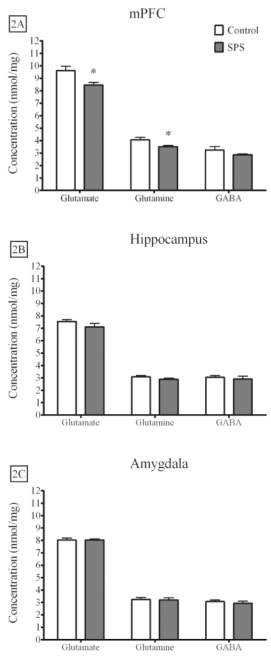

Figure 2.

The effect of SPS on glutamate, glutamine, and GABA in the mPFC, hippocampus, and amygdala complex. A) SPS attenuated basal levels of glutamate and glutamine in the mPFC, but had no effect on GABA levels. B) SPS had no effect on any neurochemicals in the hippocampus or C) amygdala complex. Data are expressed as nmol/mg tissue, presented as the mean ± standard error of the mean, and analyzed by two-tailed t-test (* $p < 0.05$). GABA – gamma amino butyric acid, mPFC – medial prefrontal cortex.

Single prolonged stress disrupts retention of extinguished fear in rats

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Research

Single prolonged stress disrupts retention of extinguished fear in rats

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Clinical research has linked post-traumatic stress disorder (PTSD) with deficits in fear extinction. However, it is not clear whether these deficits result from stress-related changes in the acquisition or retention of extinction or in the regulation of extinction memories by context, for example. In this study, we used the single prolonged stress (SPS) animal model of PTSD and fear conditioning procedures to examine the effects of prior traumatic stress on the acquisition, retention, and context-specificity of extinction. SPS administered one week prior to fear conditioning had no effect on the acquisition of fear conditioning or extinction but disrupted the retention of extinction memories for both contextual and cued fear. This SPS effect required a post-stress incubation period to manifest. The results demonstrate that SPS disrupts extinction retention, leading to enhanced fear renewal; further research is needed to identify the neurobiological processes through which SPS induces these effects.

Fear conditioning and extinction have been extensively used in recent years to study the neurobiology of psychiatric disorders characterized by excessive fear responses like phobia and post-traumatic stress disorder (PTSD) (Hofmann 2007; Hamm 2009; Koenigs and Grafman 2009; Yamamoto et al. 2009; Norrholm et al. 2010). Fear extinction refers to a form of learning that occurs when a conditioned fear stimulus (CS) no longer predicts the occurrence of an aversive event (Bouton et al. 2006; Quirk et al. 2006). It is commonly measured as a reduction in the magnitude of conditioned fear responses, including freezing behavior (Bouton et al. 2006; Quirk et al. 2006). Extinction memories are context-specific insofar as extinction retention is optimal in the context in which extinction was acquired (Bouton et al. 2006). If an extinguished CS is presented in a context that is inconsistent with the extinction context, conditioned fear returns; a phenomenon referred to as fear renewal (Corcoran and Maren 2001, 2004; Rothbaum and Davis 2003; Bouton et al. 2006).

The persistence of traumatic fear memories in PTSD suggests this disorder might be associated with extinction deficits. Previous clinical research supports this assertion (Rothbaum and Davis 2003; Anderson et al. 2004; Ressler et al. 2004; Quirk et al. 2006; Milad et al. 2008) and suggests these deficits are induced by trauma (Milad et al. 2008). However, inconsistent findings among clinical studies make it difficult to determine specific aspects of extinction that are disrupted in PTSD. Some studies report that PTSD patients have deficits in acquiring extinction as a result of enhanced fear conditioning (Peri et al. 2000; Norrholm et al. 2010), while other studies report select deficits in extinction retention with no change in fear conditioning or acquisition of extinction (Milad et al. 2008, 2009). Surprisingly, even though conditioned fear is renewed when extinction is tested outside of the extinction context (Corcoran and Maren 2001, 2004; Rothbaum and Davis 2003; Bouton et al. 2006), fear renewal has never been evaluated in PTSD patients. Thus, it is currently unclear what aspects of extinction are affected in PTSD.

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Ethical constraints make it difficult to evaluate the effects of traumatic stress on fear extinction in humans, but this relationship can be readily studied using animal models of PTSD (Armario et al. 2008). These models use stressors that induce changes in hypothalamic-pituitary-adrenal (HPA) axis function and/or anxiety behavior that mimic specific PTSD symptoms (Armario et al. 2008). Previous studies have examined the effects of trauma-like stress on certain aspects of extinction. For example, studies reported that exposing rats to predator odor (Adamec et al. 2006; Cohen et al. 2006) disrupts acquisition and retention of cued extinction in subsets of these rats (Goswami et al. 2010). The results of other work suggest that exposing rats to a single prolonged stress (SPS) (Liberzon et al. 1997, 1999; Yamamoto et al. 2009) disrupts retention of context extinction (Yamamoto et al. 2007). However, in this study, contextual fear conditioning and acquisition of contextual fear extinction were not differentiated. As a result, it is unknown if the observed contextual extinction retention deficit in stressed rats was caused by enhanced contextual fear conditioning and/or deficits in acquisition of contextual extinction. It is also possible that extinction deficits were related to the contextual regulation of extinction, including enhanced fear renewal. Therefore, we conducted the present study to evaluate the effects of trauma-like stress using the SPS animal model on multiple aspects of fear conditioning and extinction, including acquisition and retention of contextual and cued fear extinction, and fear renewal.

While several animal PTSD models (e.g., Adamec et al. 2006; Cohen et al. 2006; Armario et al. 2008), are available, we used the SPS model, because it enhances arousal (Khan and Liberzon 2004; Kohda et al. 2007) and induces changes in HPA axis function similar to those observed in PTSD patients (Yehuda et al. 1993; Liberzon et al. 1997, 1999). SPS comprises two components: a single prolonged stress episode involving the serial application of three stressors (restraint, forced swim, ether) and a quiescent period of 7 d (Liberzon et al. 1997, 1999; Yamamoto et al. 2009). The effects of the single prolonged stress episode on HPA axis function are not observed shortly after stress exposure but develop during the quiescent period (Liberzon et al. 1997, 1999). Thus, we conducted an additional experiment to determine whether the

quiescent period was also required for the development of extinction deficits.

Results

Experiment 1: SPS disrupts retention of context extinction

The design for Experiment 1 is illustrated in Figure 1A. In this experiment, we examined the effect of SPS on extinction of fear to a context that had been paired with aversive footshock. SPS was administered 1 week prior to contextual fear conditioning, which consisted of five footshock presentations in a distinct context (Context A). An analysis of variance (ANOVA) of post-shock freezing during the conditioning session revealed a significant main effect of time ($F_{(5,55)} = 19.41$, $P < 0.001$) but no main effect of stress ($F_{(1,13)} = 0.33$, $P = 0.57$) or interaction between stress and time ($F_{(5,65)} = 1.08$, $P = 0.38$). These results indicated that SPS did not affect freezing during contextual fear conditioning (Fig. 1B).

One day after fear conditioning, rats were placed into Context A for an 8-min extinction session. An ANOVA of minute by minute freezing revealed a significant main effect of time ($F_{(1,13)} = 13.32$, $P < 0.001$). There was no main effect of treatment ($F_{(1,13)} = 0.07$, $P = 0.80$) or treatment × time interaction ($F_{(7,91)} = 1.38$, $P = 0.24$). These results indicated that conditioned freezing decreased over the course of the extinction session, and there was no significant difference between SPS or control rats (Fig. 1C).

Two days after fear conditioning (and 1 d after extinction), rats were returned to Context A for an 8-min extinction test to assess the retention of extinction. An ANOVA with the factors treatment (SPS vs. control), session (extinction vs. extinction test), and time (minute 1–8) revealed a significant three-way interaction ($F_{(7,910)} = 2.55$, $P = 0.04$). This interaction was driven by significant differences in the levels of freezing across the first minute of the two extinction sessions ($t_{(13)} = 2.21$, $P < 0.05$) (Fig. 1D). This reflected the finding that freezing in SPS rats was greater at the start of the extinction test when compared to controls. These results demonstrate that SPS disrupts retention of contextual extinction.

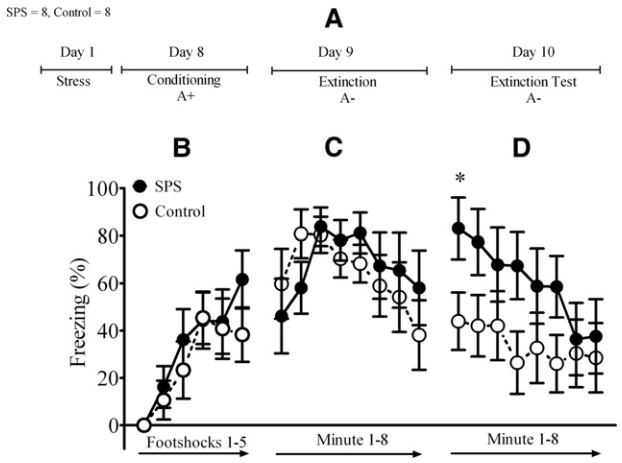

Figure 1. SPS induced deficits in contextual extinction retention. The numbers of SPS and control rats are given in the *top left* corner. (*A*) Diagram illustrates experimental design used in this study. The two character (e.g., A+) symbol describes conditioning and extinction parameters. First letter denotes context and second character denotes the presence or absence of footshocks. (*B*) SPS had no effect on freezing during conditioning or (*C*) extinction, (*D*) but disrupted contextual extinction retention. One rat was removed from the control group because it did not display a conditioned response. (*) Significant post hoc comparison between SPS and control groups at $P < 0.05$.

Experiment 2: SPS disrupts retention of extinction of cued fear

The design for Experiment 2 is illustrated in Figure 2A. In this experiment, we evaluated the effects of SPS on both the extinction and renewal of fear to an auditory CS using an ABA fear renewal paradigm (Chang et al. 2009). As in Experiment 1, SPS was administered 1 week prior to fear conditioning. On the conditioning day, rats were placed into Context A (the fear conditioning context) and subjected to five tone-shock trials. An ANOVA of cued freezing during the fear conditioning session revealed a significant main effect of trial ($F_{(5,120)} = 49.89$, $P < 0.001$). There was no significant main effect of treatment ($F_{(1,24)} = 1.07$, $P = 0.31$) or treatment × trial interaction ($F_{(5,120)} = 0.39$, $P = 0.85$), which indicated that SPS had no effect on freezing during cued conditioning (Fig. 2B).

One day after fear conditioning, rats were placed into Context B (the extinction context) for a 30-tone extinction session. An ANOVA of freezing to the auditory CS during this session revealed a significant main effect of trial (analyzed in two trial blocks) ($F_{(1,24)} = 33.85$, $P < 0.001$). Although baseline freezing in SPS rats was increased when compared to controls, there was no significant main effect of treatment ($F_{(1,24)} = 1.08$, $P = 0.31$) or a treatment × trial interaction ($F_{(15,360)} = 1.19$, $P = 0.30$) on this measure. These results indicated that conditioned fear to the CS and the extinction of that fear were not affected by SPS (Fig. 2C).

Three hours after extinction, rats were re-exposed to the fear conditioning context without tone presentations. Re-exposure to the conditioning context represents a context extinction procedure that lessens the potential confounding effect contextual conditioned freezing can have on fear renewal and is a standard procedure in ABA fear renewal paradigms (Chang et al. 2009). An ANOVA of freezing induced by re-exposure to the fear conditioning context did not reveal a significant main effect of time ($F_{(9,216)} = 0.76$, $P = 0.14$), main effect of treatment ($F_{(1,24)} = 0.07$, $P = 0.8$), or treatment × time interaction ($F_{(9,216)} = 0.65$, $P = 0.76$) (Fig. 2D).

Two days after fear conditioning and 1 d after extinction, rats were either tested for extinction retention in the extinction context or tested for fear renewal in the fear conditioning context. Freezing during this extinction retention test was analyzed using two different statistical analyses. In the first analysis, cued freezing was analyzed in two five-trial blocks, and baseline and cued freezing were analyzed using ANOVA. There was a main effect of trial blocks ($F_{(2,44)} = 83.37$, $P < 0.001$) and a significant trial blocks × context interaction ($F_{(2,44)} = 4.99$, $P = 0.01$). These results indicated that cued freezing was enhanced in the fear conditioning context when compared to the extinction context (i.e., resulting in fear renewal). There was also a main effect of treatment ($F_{(1,22)} = 16.27$, $P = 0.001$), which demonstrated that SPS enhanced freezing in both the extinction and fear conditioning contexts (Fig. 2E, top panel). However, there were no trial × treatment × testing context ($F_{(2,44)} = 0.3$, $P = 0.75$) or treatment × testing context ($F_{(1,22)} = 1.09$, $P = 0.31$) interactions. These results suggest that SPS disrupts cued extinction retention and enhances freezing during fear renewal.

In the second analysis, baseline freezing was subtracted from cued freezing, and these freezing difference scores were analyzed using ANOVA. This method has been previously used to selectively analyze the effects of experimental treatments on fear renewal (Corcoran and Maren 2004; Ji and Maren 2005). There was a main effect of testing context ($F_{(1,22)} = 4.40$, $P < 0.05$), demonstrating that cued freezing was enhanced in the fear conditioning context when compared to the extinction context (i.e., resulting in fear renewal). There was also a main effect of treatment ($F_{(1,22)} = 6.34$, $P = 0.02$), which demonstrated that SPS

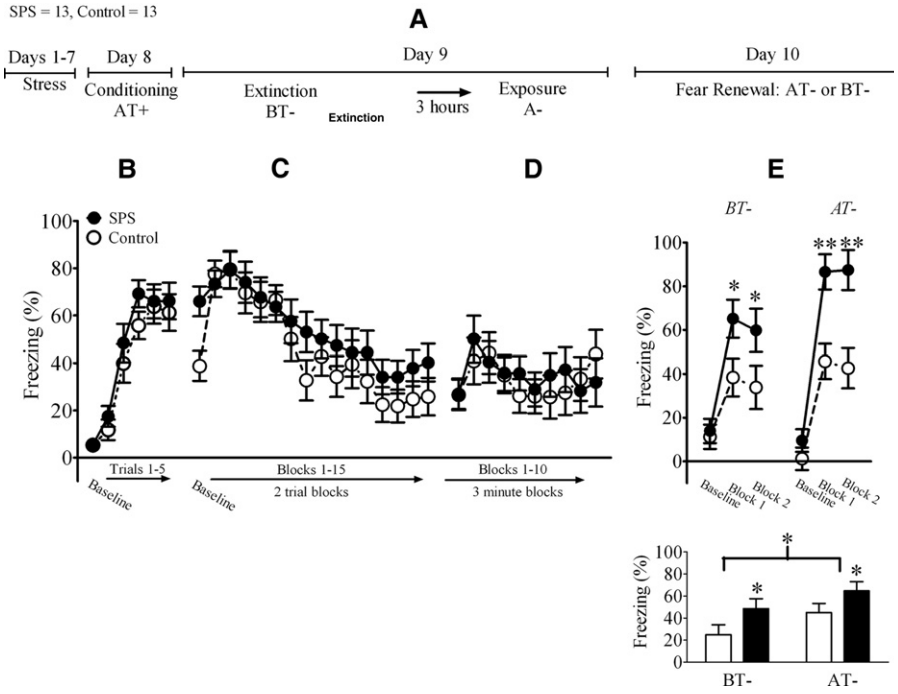

Figure 2. SPS disrupted cued extinction retention and enhanced fear renewal. (*A*) Illustrates the experimental design used in this study. "T" denotes tone presentation. (*B*) SPS had no effect on freezing during conditioning, (*C*) extinction, or (*D*) re-exposure to the conditioning context. (*E*) SPS disrupted cued extinction retention and enhanced cued freezing during fear renewal. In the *top* panel, baseline and cued freezing (analyzed in blocks of five trials) were analyzed using ANOVA. In the *bottom* panel, cued freezing was subtracted from baseline freezing and these difference scores analyzed using ANOVA. One rat was removed from the control group because it did not display a conditioned response. (*) Main effect of stress; (**) main effect of context.

enhanced cued freezing in both the extinction and fear conditioning contexts (Fig. 2E, bottom panel), but no treatment × testing context interaction ($F_{(1,22)} = 0.05$, $P = 0.82$). Taken together, these results also suggest that SPS disrupts cued extinction retention and enhances freezing during fear renewal.

Experiment 3: Extinction retention deficits induced by SPS are not observed shortly after stress exposure but develop over time

Previous research has demonstrated that the 7-d period after application of single prolonged stressors (i.e., restraint, forced swim, ether exposure) is needed to observe SPS-induced changes in HPA axis function (Liberzon et al. 1997, 1999). The aim of this experiment was to determine if a similar time interval is necessary to observe the effect of the single prolonged stressors on extinction retention.

The design for this experiment is illustrated in Figure 3A. Rats were placed into Context B (the fear conditioning context) and subjected to five tone-shock trials. An ANOVA of cued freezing during the fear conditioning session revealed a significant main effect of trial ($F_{(5,95)} = 28.78$, $P < 0.001$). There was no significant stress ($F_{(1,9)} = 0.72$, $P = 0.50$) or stress × trial interaction ($F_{(10,95)} = 0.95$, $P = 0.50$). These results demonstrated that freezing during fear conditioning was not different among the stress groups (i.e., control, SPS-1 d, SPS-7 d) (Fig. 3B).

One day after fear conditioning, a 30-tone extinction session commenced in Context A (the extinction context). An ANOVA of cued freezing during this session revealed a significant main effect of trial ($F_{(1,19)} = 7.64$, $P = 0.01$). There was no significant main effect of stress ($F_{(1,19)} = 0.19$, $P = 0.83$) or stress × trial interaction ($F_{(30,285)} = 0.83$, $P = 0.71$). These results indicated that expression of cued fear and acquisition of extinction were not different among the stress groups (Fig. 3C).

Two days after fear conditioning (and 1 d after extinction), an extinction retention test was conducted in the extinction context. An ANOVA of cued freezing during this test revealed a significant main effect of trial ($F_{(5,95)} = 28.78$, $P < 0.001$) and a treatment × trial interaction that approached significance ($F_{(2,18)} = 2.95$, $P = 0.08$). Given this potentially significant finding, we separately analyzed cued freezing during the extinction test between SPS-7 d and control rats and SPS-1 d and control rats. There was a significant main effect of treatment for SPS-7 d compared to control rats ($F_{(1,13)} = 6.25$, $P = 0.02$), but no main effect of treatment for SPS-1 d compared to control rats ($F_{(1,12)} = 0.157$, $P = 0.7$). These findings demonstrated that during the extinction test, cued freezing was enhanced in the SPS-7 d group when compared to controls, which demonstrated an extinction retention deficit in the SPS-7 d group but not in the SPS-1 d group (Fig. 3D). These findings suggest a post-stress incubation period is necessary in order to observe SPS effects on extinction retention.

Discussion

We have demonstrated deficits in contextual and cued extinction retention and enhanced fear renewal in animals exposed to SPS; an animal model of PTSD associated with enhanced arousal (Khan and Liberzon 2004; Kohda et al. 2007), altered HPA axis function (Liberzon et al. 1997, 1999), and hippocampal and medial prefrontal cortex (mPFC) abnormalities (Liberzon et al. 1999; Kohda et al. 2007; Knox et al. 2010). In contrast to these deficits, SPS exposure had no effect on acquisition or expression of conditioned fear or acquisition of conditioned extinction. Cued freezing during fear renewal was higher in SPS animals, and, insofar as extinction might have influenced renewal freezing, enhanced renewal freezing in SPS rats might have been caused by extinction retention deficits induced by SPS. However, it is also possible that a SPS-induced deficit in context processing contributed to enhanced renewal freezing in SPS rats. This is especially so because extinction retention and fear renewal were tested in two different contexts and expression of extinction during fear renewal is modulated by contextual processing (Bouton et al. 2006). Indeed, some suggestions that contextual processing abnormalities might be present both in PTSD (Liberzon and Sripada 2008; Rougemont-Bucking et al. 2011) and in SPS (Kohda et al. 2007; Yamamoto et al. 2007, 2009) have been reported in the literature. Similarly, there are a number of possible mechanisms that could contribute to the SPS extinction retention deficits we observed, such as deficits in consolidation and/or retrieval of extinction memory. A deficit in one or both memory processes could lead to a deficit in extinction retention because deficits in either of these memory processes would enhance freezing upon presentation of the extinguished CS, irrespective of the context in which

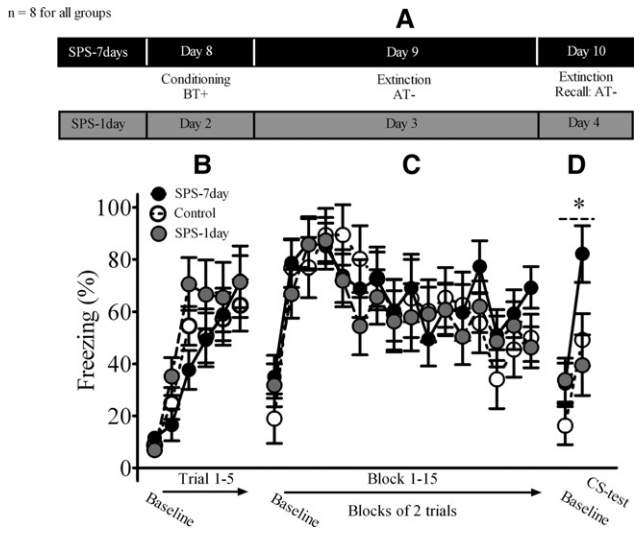

Figure 3. The effect of stress on extinction retention required a post-stress incubation period. (*A*) Illustrates the experimental design used in this experiment. (*B*) Neither the SPS-1 d nor SPS-7 d rats displayed different freezing levels during conditioning or (*C*) extinction. (*D*) Extinction retention was impaired in the SPS-7 d group, but not in the SPS-1 d group. One rat was removed from the SPS-7 d group, because its mean for the extinction test was greater than two standard deviations below the group mean. (*) Main effect of stress.

it is presented. Thus, while the results of the study clearly demonstrate that SPS disrupts extinction retention, further research will be needed to determine if extinction consolidation and/or retrieval are affected by SPS or if context processing deficits are involved in SPS enhancement of cued freezing during fear renewal.

Exposure to other kinds of stressors also induces deficits in extinction retention. These include brief uncontrollable stress (Izquierdo et al. 2006), chronic stress (Miracle et al. 2006; Garcia et al. 2008; Baran et al. 2009; Wilber et al. 2011), and footshock stress (Rau et al. 2005; Maren and Chang 2006). Also, animals that are vulnerable to the effects of stress show extinction retention deficits (Goswami et al. 2010). A comparison of SPS-induced extinction retention deficits with other types of stress-induced extinction retention deficits reveals certain similarities. For example, both SPS and chronic stress exposure alter contextual processing (Kohda et al. 2007; Yamamoto et al. 2007, 2009; Baran et al. 2009), and these changes in contextual processing might contribute to chronic stress-induced extinction retention deficits (Baran et al. 2009) and SPS-enhanced fear renewal (see above). However, there are also notable differences. Exposing animals to brief uncontrollable and chronic stress prior to fear conditioning, or conducting fear conditioning in animals that are vulnerable to stress, enhances cued conditioned responding during fear conditioning and/or fear extinction (Izquierdo et al. 2006; Miracle et al. 2006; Goswami et al. 2010; Wilber et al. 2011), which suggests that stress-induced changes in fear memory may contribute to changes in extinction retention. This interpretation is also supported by the observation that, when the cue and the footshock presentations are not explicitly paired during fear conditioning (i.e., pseudoconditioning), chronic stress pre-exposure has no effect on extinction retention (Baran et al. 2009; Wilber et al. 2011). SPS exposure prior to fear conditioning disrupted extinction retention without having any effects on acquisition or expression of conditioned fear. While this suggests that SPS exposure disrupts extinction retention without affecting fear memory expression, further research is needed to explicitly test this.

Neurobiology of extinction retention deficits

Exposure to SPS induced deficits in the retention of both cued and contextual extinction in our animals, and there are number of specific neurobiological mechanisms that could mediate these effects. With respect to specific brain regions involved in extinction retention, previous research has demonstrated that the infralimbic (IL) region of the medial prefrontal cortex (mPFC) is critical for this. Temporary inactivation of the IL cortex disrupts acquisition of extinction (Sierra-Mercado et al. 2006); N-Methyl-D-aspartic receptor blockade in the IL cortex disrupts acquisition and consolidation of extinction (Burgos-Robles et al. 2007; Sotres-Bayon et al. 2009); stimulation of the IL enhances extinction retention (Vidal-Gonzalez et al. 2006); and permanent IL cortical lesions disrupt extinction retrieval (Lebron et al. 2004). It is currently believed that the IL cortex modulates extinction retention by modulating neural activity in brain regions critical for the expression of conditioned fear, such as the intercalated region, basolateral complex, and central nucleus of the amygdala (Rosenkranz and Grace 2002; Rosenkranz et al. 2003; Pare et al. 2004; Quirk and Mueller 2008; Li et al. 2011).

Studies that have specifically focused on the neurobiology of stress-induced extinction retention deficits also suggested that stress-induced changes in IL cortical function may underlie stress-induced extinction retention deficits (Izquierdo et al. 2006; Baran et al. 2009; Wilber et al. 2011). For example, stress-induced retraction of apical dendrites in the IL is associated with extinction retention deficits (Izquierdo et al. 2006; Miracle et al. 2006), and rats exposed to chronic stress show deficits in extinction retention and fail to show an enhancement in single unit activity in the IL cortex during extinction retention testing (Wilber et al. 2011). On the molecular level, repeated stress exposure enhances corticosterone-glucocorticoid receptor (GR) binding (Meaney et al. 1985; Xu et al. 1998; Liu and Aghajanian 2008; Gourley et al. 2009) and excitatory neurotransmitter release (Moghaddam 1993; Martin and Wellman 2011), which then can disrupt IL function (McEwen 2001; Miracle et al. 2006; Liu and Aghajanian 2008; Wilber et al. 2011). While there are clearly differences between repeated stress exposure within chronic stress procedures and SPS, there may yet be intermediate outcomes (e.g., enhanced GR signaling), by which chronic stress and SPS induce extinction retention deficits. For example, SPS does not alter baseline or stress-enhanced corticosterone levels (Liberzon et al. 1997; laboratory observation) but enhances GR expression in emotional circuits in the brain (Liberzon et al. 1999; Stout et al. 2010), including the PFC (Knox et al. 2011). SPS-enhanced GR expression in the PFC may serve to enhance corticosterone-GR binding in the IL cortex, which may disrupt IL cortical function, thereby inducing extinction retention deficits. This interpretation is indirectly supported by the finding that both SPS extinction retention deficits and GR enhancement require a similar post-stress incubation period to manifest (Liberzon et al. 1999; Experiment 3). Thus, SPS extinction retention deficits may not be observed one day after stress exposure (Experiment 3), because enhanced GR expression in the mPFC has not occurred at this point in time.

Alternatively, SPS effects on extinction retention can be mediated through SPS-induced changes in excitatory neurotransmitter levels, as SPS exposure attenuates glutamate levels in the mPFC (Knox et al. 2010). If SPS effects on glutamate levels in the IL cortex reflect the physiological status of glutamatergic neurotransmission in these animals, then this could result in decreased excitatory tone in the IL cortex, which, in turn, could directly affect extinction retention by disrupting IL cortical modulation of downstream targets, such as the intercalated region of the amygdala (Rosenkranz and Grace 2002; Rosenkranz et al. 2003; Pare et al. 2004; Quirk et al. 2006; Li et al. 2011). It has

been proposed also that an "amygdala kindling mechanism" may mediate footshock-induced extinction retention deficits (Rau et al. 2005). We find no evidence of amygdala involvement in SPS effects on extinction retention (Knox et al. 2010). If amygdala kindling is associated with footshock-induced extinction retention deficits, SPS and footshock stress induce extinction retention deficits via different neurobiological mechanisms. Thus, SPS may induce extinction retention deficits by enhancing GR expression and/or decreasing glutamate levels in the IL region of the mPFC, but further research is needed to explore these possibilities.

Unexpected findings and potential limitations

We found no freezing difference between SPS and control rats during the baseline period of the fear renewal test. This might seem inconsistent with the contextual extinction retention deficit we have observed in Experiment 1, because the baseline period during renewal also reflects contextual extinction retention. However, the duration of the contextual extinction sessions differed greatly between the two experiments (8 min in Experiment 1, 30 min in Experiment 2). This procedural difference may explain the apparently contradictory findings and may also suggest that increasing extinction training for SPS animals might overcome the observed contextual extinction deficits. This hypothesis and the additional possibility that increasing cued extinction training may also attenuate cued extinction retention deficits induced by SPS should be explicitly addressed by future research.

In this study, animals exposed to SPS developed extinction retention deficits as a group, but only a proportion of individuals that experience trauma develop PTSD (Kessler et al. 1995; Yehuda and LeDoux 2007). Indeed, there is also variability in animal responses to SPS exposure (see Standard Errors). However, additional studies will be required to directly test this hypothesis. Combining SPS with other experimental manipulations (e.g., exposing genetically susceptible strains of animals to SPS) or increasing the number of rats exposed to SPS and developing a criteria for selecting rats that are most affected by SPS (Cohen et al. 2005) might be used to address these important questions.

Summary

Previous clinical studies suggested that trauma exposure induced selective deficits in extinction retention in PTSD patients (Milad et al. 2008, 2009). The results of our study using the PTSD animal model SPS further supports this hypothesis, as we have found similar, newly acquired extinction retention deficits in animals exposed to SPS treatment. Detailed examination of fear conditioning and extinction also revealed evidence of enhanced fear renewal in SPS exposed animals, a finding that can be directly caused by SPS extinction retention deficit, or, alternatively, suggests context processing deficits in SPS animals. Our time line experiments further suggest that trauma-induced deficits in extinction retention may require a post-trauma incubation period to manifest. Previous SPS studies suggest possible mechanisms that could mediate SPS extinction retention deficits and fear renewal enhancement, such as increased GR expression and/or decreased glutamatergic signaling in the IL. However, additional research is required to address these questions empirically.

Materials and Methods

Subjects

The subjects were 68 adult male Sprague Dawley rats (42–45-d-old; 150 g), obtained from Charles River (Wilmington, MA). Upon arrival, all rats were pair-housed for a minimum of 3 d and were then individually housed after exposure to stress or a control procedure. All rats had ad libitum access to water and standard rat chow. All experimental procedures were approved by the Veteran Affairs Institutional Animal Care Usage Committee.

SPS

Prior to conditioning, the rats were assigned to a stress or control procedure. Rats in the stress group were exposed to restraint for 2 h, followed immediately by 20 min of forced swimming. Forced swimming occurred in a plastic tub (55.6-cm diameter, 45.4-cm height), filled two-thirds from the bottom with water (20–24°C). Fifteen minutes after the forced swim, rats were exposed to ether (75 mL) in a glass dessicator until they were fully anesthetized displaying no toe or tail pinch response (<5 min of ether exposure). Immediately after the induction of general anesthesia, rats were removed from the dessicator, housed singly, and left undisturbed for either 1 d (SPS-1 d) or 7 d (SPS-7 d). Rats assigned to the control group were housed singly, left undisturbed, and remained in the housing colony until experimental procedures commenced.

Behavioral apparatus

All sessions were conducted in eight identical rodent observation chambers constructed of aluminum and Plexiglas (30 × 24 × 21 cm; MED Associates), situated in sound-attenuating chambers and located in an isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center). The grid floor was connected to a shock source and a solid-state grid scrambler (MED Associates) which delivered the footshock unconditioned stimulus (UCS). Mounted on one wall of the chamber was a speaker to provide a distinct auditory CS; on the opposite wall was a 15-W house light and a fan, which provided background noise (65 dB). Cameras mounted to the ceiling of the sound-attenuating chambers were used to record behavior, which was scored offline.

Two unique contexts were created by manipulating auditory, visual, and olfactory cues: Context A comprised a 1% acetic acid solution placed in trays at the bottom of the chambers, the house light on, chamber doors closed, and fans on in the chambers; Context B comprised a 1% ammonium hydroxide solution in chambers, red light on, chamber doors open, and fans off.

Experiment 1: Contextual fear conditioning, extinction, and extinction retention

On Day 1, 16 rats (SPS = 8; control = 8) were transported from their home cages in squads of eight and placed in the conditioning context (Context A). Rats received five unsignaled footshocks (1.0 mA, 1 sec) beginning 210 sec after being placed in the chambers. There was a 60-sec inter-trial interval (ITI), and the rats remained in the chambers for 60 sec after the last footshock presentation. One day after conditioning, all rats were placed back into Context A for 8 min without any presentations of the US in order to extinguish fear responding to the context. Two days after conditioning, all rats were placed into Context A for 8 min to test extinction.

Experiment 2: Cued fear conditioning, extinction, and fear renewal

A separate group of 28 rats (SPS = 14, control = 14) were placed in Context A and received five paired presentations of a tone (10 sec, 2 kHz, 80 dB) that coterminated with a footshock (1.0 mA, 1 sec) beginning 180 sec after being placed in Context A. There was a 60-sec ITI, and the rats remained in the chambers for 60 sec after the last footshock presentation. One day after conditioning, all rats were placed into a novel context (Context B) and were presented with 30 tone presentations (10 sec, 2 kHz, 80 dB, 60-sec ITI), in the absence of footshock, beginning 180 sec after being placed into the chambers in order to extinguish fear responding to the tone (i.e., extinction training). Three hours following

extinction training, all rats were re-exposed to Context A for 30 min without any stimuli presentations. Two days after conditioning, rats were placed into the extinction context (Context B; SPS = 6, control = 6) or the conditioning context (Context A; SPS = 8, control = 8) and were presented with 10 tones beginning 180 sec after being placed into the chambers in order to assess extinction retention in these contexts.

Experiment 3: Cued fear conditioning, extinction, and extinction retention

Prior to fear conditioning, 16 rats were exposed to SPS and left undisturbed for either 7 d (SPS-7 d, $n = 8$), as in the previous experiments, or 1 d (SPS-1 d, $n = 8$). Another group of eight rats were assigned to the control condition. Rats were placed in Context B and fear conditioned to a tone cue as described above. One day after conditioning, all rats were placed into a novel context (Context A) and were presented with 30 tones (10 sec, 2 kHz, 80 dB, 60-sec ITI) beginning 180 sec after being placed into the chambers in order to extinguish fear responding to the tone. Two days after conditioning, all rats were placed back into the extinction context (Context A) and were presented with two tones beginning 180 sec after being placed into Context A in order to assess cued extinction retention.

Data analysis and statistical analysis

Freezing was defined as the absence of movement, except that necessary for breathing, for >2 sec and quantified as a percentage of the total time recorded. These values were analyzed using ANOVA, and post hoc comparisons using *t*-tests, with a Bonferroni correction, were performed when significant overall F ratios were obtained. The criterion for significance was set at $P < 0.05$. Rats that did not show a conditioned freezing response >30% at the start of a fear extinction session were excluded from final analyses. In addition, rats exhibiting freezing levels ±2 standard deviations from a group mean were removed from the analyses. All data are represented as means ± SEM.

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Unconditioned freezing is enhanced in an appetitive context: Implications for the contextual dependency of unconditioned fear

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ABSTRACT

It has been well established that expression of conditioned fear is context independent, but the context dependency of unconditioned fear expression has rarely been explored. A recent study reported that unconditioned freezing in rats is enhanced in a familiar context, which suggests that unconditioned fear expression can be modulated by contextual processing. In order to further explore this possibility we examined unconditioned freezing in novel, familiar, and appetitive contexts; and attempted to identify brain regions critical for context-related changes in unconditioned freezing by measuring c-Fos mRNA levels in emotional circuits. Unconditioned freezing was enhanced in the appetitive context, and this enhancement was accompanied by increased c-Fos mRNA expression in the medial amygdala and hippocampus, but attenuated expression in the medial prefrontal cortex. In the appetitive context, expectation of a reward coupled with detection of threat may have enhanced unconditioned fear expression, which suggests that unconditioned fear expression can be modulated by contextual factors. Context-related expectancy mismatch may explain the enhancement of unconditioned fear expression seen in this study and warrants further examination.

1. Introduction

Previous research has demonstrated that conditioned fear, prior to extinction learning, is robustly expressed outside of the context in which fear conditioning was acquired (Bouton & King, 1983; Corcoran, Desmond, Frey, & Maren, 2005; Corcoran & Maren, 2001; Ji & Maren, 2005; Knox & Berntson, 2006). This suggests that expression of conditioned fear is independent of the context in which it is learned (i.e. context independent), but research examining the effects of context on unconditioned fear has rarely been conducted. Previous studies have demonstrated trimethylthiazoline (TMT, a component of fox feces) induces defense responses that are indicative of unconditioned fear (Endres, Apfelbach, & Fendt, 2005; Wallace & Rosen, 2000), and the results of a recent study suggest that TMT-induced freezing is enhanced when TMT is presented in a context to which rats have been pre-exposed (i.e. familiar context) (Nikaido & Nakashima, 2009). The finding that TMT-induced freezing is enhanced in a familiar context thus suggests that unconditioned fear expression, unlike conditioned fear expression, can depend on the context in which unconditioned fear is induced. If so, then sensitivity to context-related modulation may be an important feature that differentiates conditioned and unconditioned fear expression; and understanding this differentiation may help to advance understanding of the neurobiology of these emotions.

The idea that context familiarity enhances fear may at first seem counter-intuitive. However, context familiarity is created by repeated, non-threatening exposure that creates a safety-related contextual memory, which in turn creates a non-aversive expectation in this context. When unexpectedly, a threat is detected in the context previously perceived as safe, this context-related expectancy mismatch may enhance unconditioned fear expression. Indeed, enhancement of anxiety/fear caused by a mismatch between expectation of a reward or safety in a context, but detection of threat in the same context (i.e. enhanced conflict) is predicted by neurobiological theories of anxiety (Gray & McNaughton, 2000; McNaughton & Corr, 2004). While this hypothesis is plausible, alternative explanations can account for enhanced TMT-induced freezing in a familiar context. Repeated exposure to the same context may result in a decrease in locomotor activity, which might be indistinguishable from freezing (i.e. false positive). This line of reasoning suggests enhanced TMT freezing in a familiar context might represent habituation of locomotor activity rather than fear-induced freezing. If this is indeed true, one would not expect enhanced neural processing in fear circuitry to accompany

Abbreviations: TMT, trimethylthiazoline; BSNT, bed nucleus of the stria terminalis; MeA, medial amygdala; PL, prelimbic cortex; mPFC, medial prefrontal cortex; β-ME, β-mercaptoethanol; DG, dentate gyrus.

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TMT-induced freezing in a familiar context. Simultaneous examination of the freezing and neural activity in fear circuitry should help to disambiguate behavioral findings supporting one of these alternative interpretations.

To test these competing alternatives, we examined TMT-induced freezing in a novel context, familiar context, a context reinforced with food reward (i.e. appetitive context), and TMT-induced c-Fos mRNA expression in these contexts. Using expectancy mismatch logic, reinforcing a context with food should create an expectation of a rewarding context. If a threat (i.e. TMT) is presented in the same context, this context-related expectancy mismatch should enhance unconditioned fear expression as compared to the similarly familiar, but not necessarily rewarding, context and novel context. Thus, if unconditioned fear is enhanced because of context-related expectancy mismatch, then TMT-induced freezing should be highest in the appetitive context: the only context that was reinforced with a specific non-aversive outcome (i.e. food reward). On the other hand, if a reduction in locomotor activity due to familiarity is the only contributing factor, then TMT-induced freezing should be similarly high in both the familiar and appetitive contexts. Furthermore, if TMT-induced freezing is due to a hypothesized context-expectancy mismatch, then evidence of altered neural activity would be expected in brain regions involved in: (a) context processing, i.e. hippocampus (Anagnostaras, Gale, & Fanselow, 2001; Maren, 2001; Mizumori, Ragozzino, Cooper, & Leutgeb, 1999; Myers & Gluck, 1994; Smith & Mizumori, 2006; (b) expression of TMT-induced freezing i.e. bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA) (Fendt, Endres, & Apfelbach, 2003; Muller & Fendt, 2006; (c) inhibition of TMT-induced freezing i.e. prelimbic region (PL) of the medial prefrontal cortex (mPFC) (Fitzpatrick, Knox, & Liberzon, 2011). Finally, we also measured circulating corticosterone levels after TMT exposure in our animals, as indices of stress reactivity. Thus, we examined behavioral, neural, and stress axis responses to TMT in three different contexts, comparing these responses to similar responses induced by β-mercaptoethanol (β-ME, a noxious but non-threatening fluid), to control for the presence of aversive, but non-fear-specific stimuli.

2. Material and methods

2.1. Animals

Fifty-two male Sprague Dawley rats from Charles River (Wilmington MA) were used. All rats were pair-housed at the Veterinary Medical Unit of the Ann Arbor Veterans Affairs (VA) Medical Center, maintained on a 12:12 h light/dark cycle, and handled at least three times before commencing any experimental procedure. Room temperature (19–21 °C) and humidity (50 ± 10%) were tightly controlled, and food and water were available *ad libitum*. All experimental procedures were approved by the VA Institutional Animal Care Usage Committee and in compliance with National Institute of Health guidelines for the treatment of animals.

2.2. Contextual procedures and fluid presentation

All behavioral tests were conducted between 9:00 a.m. and 12:00 p.m. The context for all behavioral tests was a testing arena that was a cube with 30.5 cm unit length. For the novel context treatment (TMT = 7, β-ME = 6), the freezing test session was conducted in the testing arena, which the rats had never before been exposed to. For the familiar context treatment (TMT = 7, β-ME = 6), rats were exposed to the context for 10 min a day over a 5 day period. The appetitive context treatment (TMT = 7, β-ME = 6) was similar to the familiar context treatment, except that during each 10 min exposure, 10 palatable treats (fruit loops) were presented in the testing arena. At no time were rats' food deprived. To initiate the freezing test session, either 15 μL of TMT (in neat form) or β-ME was presented in novel, familiar, or appetitive contexts on a Kim Wipe secured to the floor of the arena. Behavior was then recorded for 10 min and scored at a later date.

2.3. C-Fos mRNA in situ hybridization

After the freezing test session, rats were removed from the testing arena and isolated for 15 min in their home cages. This was done in order to allow for upregulation of c-Fos mRNA (see below). We did not continuously expose rats to TMT for 25 min, because we were only interested in TMT changes in neural activity that corresponded with changes in TMT-induced freezing. TMT-induced freezing, when compared to other noxious, non-aversive fluids, is robustly observed within the first 10 min of fluid exposure. After this, decreases in locomotor activity can result in behavior that looks like freezing, which makes it difficult to differentiate TMT fear behavior from decreases in locomotion that occur as a result of the animal being in the testing arena for an extended period of time (laboratory observation; Wallace & Rosen, 2000). Thus, the freezing test session was 10 min and brains were obtained 15 min after the end of the freezing test session.

After rapid decapitation, brains were placed in isopentane that was chilled to −20 °C, and transferred to a −80 °C freezer until further processing. Details of c-Fos in situ hybridization protocol are similar to those utilized by Day and colleagues (Day, Masini, & Campeau, 2004) and are only briefly described here. Sections through brain regions were taken using a cryostat (Leica, Bannockburn IL), thaw mounted onto superfrost slides, and stored in a −80 °C freezer until further processing. Adjacent sections were also taken and treated to visualize Nissl substance in order to aid in the visualization of brain anatomical substrates. In order to conduct in situ hybridization, sections were fixed in a 4% paraformaldehyde solution for 2 h and rinsed in standard saline citrate (SSC) buffer. The slides were then acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min and dehydrated in a progressive series of alcohols.35S-labeled cRNA probes were generated for c-Fos from cDNA subclones in transcription vectors using standard in vitro transcription methodology. The rat c-Fos cDNA clone (courtesy of Dr. T. Curran, St. Jude Children's Research Hospital, Memphis, TN) was subcloned in pGem3Z and cut with HindIII. Riboprobes were then labeled in a reaction mixture consisting of approximately 1 μg linearized plasmid, 4 μL T7 transcription buffer (Promega, Madison WI), 4 μL of 800 mCi/mL of 35S-UTP (PerkinElmer, Waltham MA), 4 mM NTPs (CTP, ATP, and GTP), 10 mM dithiothreitol, 20 U RNAse inhibitor, and 14 U RNA polymerase (T7). The reaction was allowed to proceed for 1 h at 37 °C. After this, 20U of a RNAse-free DNAse was then added to the reaction mixture for 15 min at room temperature. Riboprobe was separated from free nucleotides and proteins over a Sephadex G50–50 column and 1 μL counted in a scintillation counter. Only riboprobes that were labeled with at least 1 million dpms of radioactivity per micro liter were used for hybridization experiments. Riboprobes were diluted in 50% formamide hybridization buffer (Amnesco, Solon OH) to yield approximately 1×10^6 dpm/100 μL of buffer. Hybridization buffer (100 μL) was applied to each slide and sections were coverslipped. Slides were placed in sealed plastic boxes lined with filter paper moistened with 50% formamide, and were subsequently incubated overnight at 55 °C. Coverslips were then removed, and slides were rinsed several times in 2 × SSC, incubated in RNAse A (Sigma–Aldrich, St. Louis MO) buffer (60 μg/mL) at 37 °C for 30 min, and washed successively in decreasing concentrations of SSC for 5 min each. Sections were then washed in 0.1 × SSC for 60 min at 65 °C. Slides were subsequently dehydrated in a graded series of alcohols, and exposed to Kodak MR X-ray film for 10–20 days.

2.4. Corticosterone assay

After rapid decapitation, trunk blood was collected in EDTA coated tubes, and centrifuged at 1000*g* for 20 min. Plasma was collected and then stored at −80 °C until assayed. Corticosterone was assayed using a corticosterone kit (tkrc1) in accordance with the manufacturer's instructions (Siemens, Los Angeles CA). Baseline levels of plasma corticosterone were established by assaying plasma corticosterone in rats that were immediately removed from the housing colony ($n = 13$).

2.5. Data processing and statistical analysis

The freezing test session was blocked into 5, 2-min periods. Freezing was defined as total cessation of locomotion, expressed as a percentage of the total time in each period, and subjected to a three factors ANOVA, with odor treatment as the first factor (TMT vs. β-ME), context (novel, familiar, appetitive) as the second factor and time (T1–5) as the third factor. Freezing was scored by one individual, and freezing scores from this individual were then verified by two others blind to group assignments.

To determine c-Fos mRNA levels, sections on film were digitized using a SCION 10 bit Crystal Clear Display camera (SCION, Frederick MA) with an attached 50 mm Mega Pixel fixed C-mount lens. The darkness values of digitized images were compared to a linear scale of darkness values in order to ensure that the range of darkness values obtained on film were not outside of the linear range of darkness values. Mean gray values were obtained in the following manner. All digital images had background noise subtracted using a 2D rolling ball procedure with a rolling ball radius of 50 pixels. Signal pixels of a region of interest were defined as being three standard deviations above the mean gray value of five arbitrarily defined cell poor areas adjacent to the region of interest. The mean gray values in the regions of interest were then calculated. Four to eight measurements for each brain region were averaged to get a single integrated mean gray value per region. Delineation of the regions of interest was determined using Nissl stained adjacent sections, so that the same regions were assessed in all brains. Mean gray values for the PL cortex, infralimbic cortex (IL), MeA, and BNST were analyzed using a two factor design with odor treatment as the first factor and context as the second factor. For analysis of hippocampal c-Fos mRNA expression, the hippocampus was separated into CA and DG regions and analyzed using a three factors design with odor treatment as the first factor, context as the second factor, and brain region (CA, DG) as the third factor.

Plasma corticosterone levels were calculated using the manufacturer's instructions. In order to determine if presentation of TMT and β-ME induced a corticosterone response, baseline corticosterone levels were compared against corticosterone levels in rats exposed to TMT and β-ME, in all contexts, and subjected to one way ANOVA (baseline, TMT, β-ME). Changes in corticosterone response across context were analyzed using a two factors design, with odor treatment as the first factor and context as the second factor.

For all statistical analyses, main and simple effects were analyzed using ANOVA, while main and simple comparisons were analyzed using *t*-test with Bonferroni correction applied where necessary. The criterion for significance was set at $p < .05$.

3. Results

3.1. TMT enhances freezing in an appetitive context

Exposure to TMT, as compared to β-ME, led to enhanced freezing (main effect of odor: $F_{(1,33)} = 31.02$, $p < .001$). There was no main effect of context ($F_{(2,33)} = 1.93$, $p = .16$), but context effects differed depending upon treatment (significant odor × context interaction; $F_{(2,33)} = 4.18$, $p = .024$). With β-ME exposure, freezing levels were equal and low in all three contexts; with TMT exposure, freezing developed more rapidly and dramatically in the appetitive context than in the novel or familiar contexts (Fig. 1A). Also, TMT-induced freezing was equivalent in the novel and familiar contexts, but significantly elevated in the appetitive context – with a main effect of context for TMT-induced freezing ($F_{(2,18)} = 3.9$, $p = 0.039$; Fig. 1A, second panel) and a significant post-hoc comparison (Appetitive vs. (Familiar and Novel); $t_{(12)} = 2.46$, $p = .03$; Fig. 1A and 1B).

3.2. Presentation of TMT in the appetitive context enhances c-Fos mRNA expression in the hippocampus and MeA, but not mPFC

Both TMT and β-ME presentation induced c-Fos mRNA expression in the hippocampus (dentate gyrus (DG) and CA regions), MeA, PL, and (IL) cortex. C-Fos mRNA in these regions could be reliably observed after 10 days of film exposure, but c-Fos mRNA was at undetectable levels in the BNST even after 20 days of film exposure (Fig. 2). As a result, statistical analyses could not be conducted on BNST c-Fos mRNA levels.

In the familiar environment, there were no differential effects of TMT vs. β-ME on c-Fos mRNA levels in any brain region (Fig. 3A), with no main or interaction effects in an omnibus ANOVA across all brain regions examined (odor treatment: $F_{(1,9)} = 1.004$, $p = .343$; odor treatment × brain region: $F_{(4,36)} = 1.207$, $p = .325$). We subsequently examined treatment effects in the novel and appetitive contexts, examining odor treatment (TMT vs. β-ME) and context (novel vs. appetitive) effects using separate two factor ANOVAs for each region of interest.

Consistent with fear-specific effects of the TMT, in both hippocampus (DG and CA) and MeA, TMT increased c-Fos mRNA levels relative to β-ME in both novel and appetitive contexts (Fig. 3B, main effects of odor: $F_{(1,33)} = 6.081$, $p = .019$ and $F_{(1,21)} = 36.07$, $p < .001$ for hippocampus and MeA, respectively), with no effect of context ($F_{(2,33)} = 1.773$, $p = .186$ and $F_{(1,21)} = 2.297$, $p = .144$ for hippocampus and MeA, respectively), and no odor treatment × context interactions ($F_{(2,33)} = 1.467$, $p = .245$ and $F_{(1,21)} = .333$, $p = .57$ for hippocampus and MeA, respectively). There was an overall subregion difference within hippocampus – c-Fos mRNA expression was greater in DG than CA regions ($F_{(1,33)} = 20.499$, $p < .001$); but there were no differences between hippocampal subregions in odor treatment or context effects (all interactions were non-significant: odor × region $F_{(1,33)} = 1.602$, $p = .214$, context × region $F_{(2,33)} = .459$, $p = .636$, odor × context × region $F_{(2,33)} = 2.107$, $p = .138$). In contrast, there were both odor treatment and context effects in both mPFC regions. TMT significantly increased c-Fos mRNA expression relative to β-ME in both the PL and IL cortices (main effect of odor: $F_{(1,20)} = 16.49, p = .001$; $F_{(1,20)} = 21.16, p < .001$, for PL and IL respectively), but this enhancement was only evident in the novel context and was strikingly absent in the appetitive context (Fig. 3C). For the PL, there was no main effects of context ($F_{(1,20)} = .731, p = .403$), but there was a significant odor treatment × context interaction ($F_{(1,20)} = 8.304, p = .009$), due to increased c-Fos mRNA expression by TMT relative to β-ME in the novel ($t_{(10)} = 6.336, p < .001$), but not the appetitive ($t_{(10)} = .705, p = .497$), context. In fact, TMT c-Fos mRNA levels were significantly lower in the appetitive compared to the novel context, ($t_{(12)} = 2.776, p = .034$). For the IL, the main effect of context ($F_{(1,20)} = 6.027\ 1, p = .022$) and the odor treatment × context interaction ($F_{(1,20)} = 4.944, p = .038$) were both significant. As in PL, these effects were due to a significant elevation of c-Fos mRNA expression by TMT relative to β-ME in the novel context ($t_{(10)} = 4.903, p = .001$), without a TMT effect in the appetitive context ($t_{(10)} = 1.655, p = .129$), and with significantly lower c-Fos mRNA expression in the

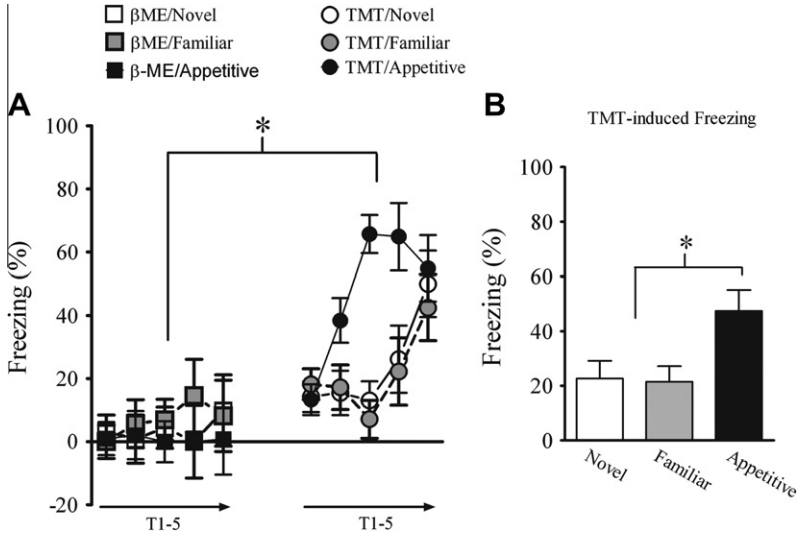

Fig. 1. TMT-induced freezing is enhanced in an appetitive context. (A) TMT-induced freezing was enhanced when compared to β-ME freezing. (B) TMT-induced freezing across the entire test session was enhanced in the appetitive context when compared to freezing in novel and familiar contexts. * – significant comparison for $p < .05$.

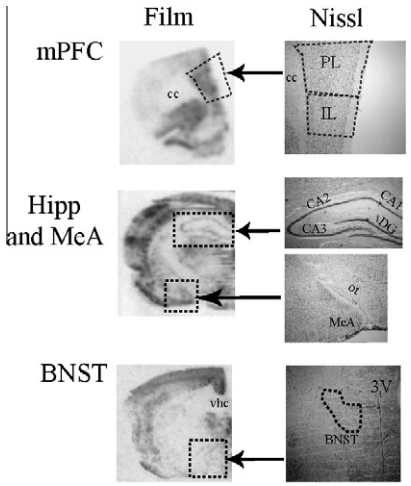

Fig. 2. Representative sections demonstrating c-Fos mRNA expression in this study. TMT and β-ME presentation induced c-Fos mRNA expression in all brain regions analyzed except the BNST. Nissl sections were taken with an Olympus CX41 (Center Valley PA) microscope at a magnification of 4X. 3 V – third ventricle, BNST – bed nucleus of the stria terminalis, cc – corpus callosum, DG – dentate gyrus, Hipp – hippocampus, IL – infralimbic cortex, MeA – medial amygdala, mPFC – medial prefrontal cortex, ot – optic tract, PL – prelimbic cortex, vhc – ventral hippocampal commissure.

appetitive as compared to the novel context with TMT exposure ($t_{(12)} = 3.031, p = .02$).

3.3. TMT corticosterone responses

Corticosterone levels were significantly elevated during TMT and β-ME exposure relative to baseline ($F_{(2,49)} = 15.35, p < .001$; Fig. 4A). Presentation of TMT and β-ME produced significant elevations in corticosterone levels ($t_{(32)} = 6.16, p < .001$ and $t_{(28)} = 4.94, p < .001$ for TMT and β-ME, respectively; Fig. 4A). Corticosterone responses did not differ between these odor treatments ($F_{(1,32)} = .196, p = .661$) and were not significantly affected by context (context: $F_{(2,32)} = .264, p = .77$; odor × context interaction: $F_{(2,32)} = 2.74, p = .08$; Fig. 4B); though the trend level interaction term and Fig. 4 provide a hint that TMT might elicit a greater corticosterone response than β-ME in the familiar environment alone.

4. Discussion

4.1. Context-related expectancy mismatch enhances unconditioned fear

Even though TMT and β-ME are both noxious odorants, only TMT induced consistent freezing behavior. Indeed, β-ME-induced freezing was minimal. These observations are consistent with previous studies that have demonstrated TMT has threat-specific properties (Endres et al., 2005; Wallace & Rosen, 2000), and the assertion that TMT-induced freezing is indicative of unconditioned fear. Both initial and overall levels of TMT-induced freezing in the appetitive context were enhanced when compared to TMT-induced freezing in the novel and familiar contexts. Even though TMT-induced freezing was equivalent at the end of the freezing test session in all contexts, freezing levels at this point in time were at the high end of freezing behavior induced by TMT exposure (Knox & Berntson, 2006; Wallace & Rosen, 2000). Equivalence in freezing across groups at this time point may reflect a ceiling effect.

Pairing food reward with a context may have resulted in formation of a context-reward memory that generated an expectation of reward in the appetitive context. When rats were returned to the appetitive context, presentation of TMT may have resulted in a context-related expectancy mismatch (i.e. expect reward, but detect threat in context), which enhanced fear expression even though the fear itself was unconditioned. This enhancement of unconditioned fear by prior experience within the context suggests that expression of unconditioned fear can be modulated by contextual processing.

At least one prior report has documented context modulation of TMT-induced freezing (Nikaido & Nakashima, 2009). In that report, TMT-induced freezing was enhanced in a familiar context, whereas in our study the appetitive, but not the familiar context, produced enhancement of TMT-induced freezing. Though Nikaido and Nakashima (2009) did not interpret their result in terms of contextual modulation of unconditioned fear, their data are consistent with this idea and they do highlight the influence of external environment in this, "innate fear" model. The apparent discrepancy between their study and ours in the type of context found to modulate fear expression is likely due to a key design difference. In their design, the familiar context for testing was the home cage, so the animals were exposed to TMT in the same cage in which they had been fed and watered. The novel context was an identical

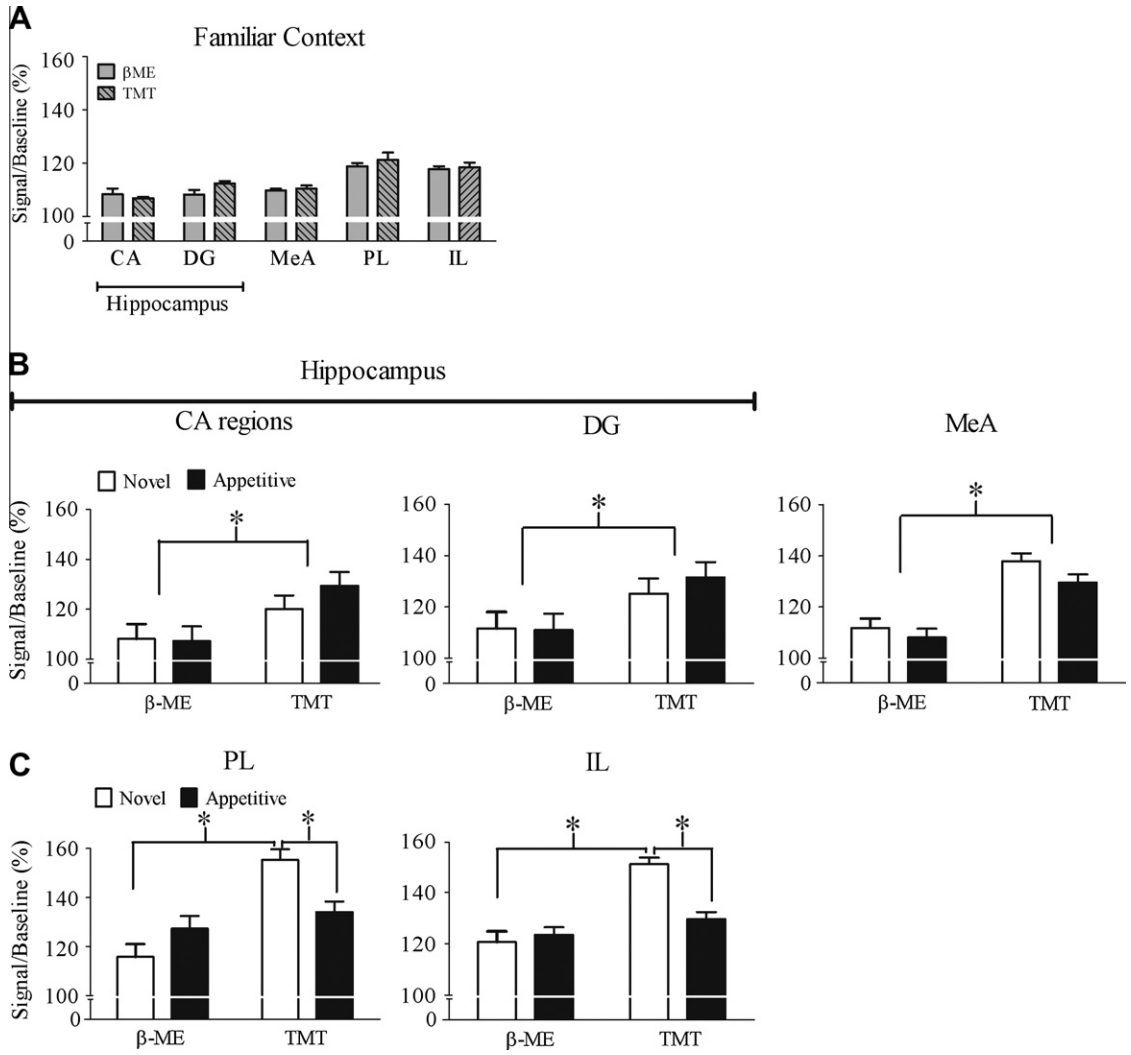

Fig. 3. TMT presentation in the appetitive context enhances c-Fos mRNA expression in the hippocampus and MeA, but not in the mPFC. C-Fos mRNA levels are expressed as a percent change from baseline. (A) In the familiar context, TMT did not enhance c-Fos expression in comparison to β-ME in any brain region. (B) TMT enhanced hippocampal and MeA c-Fos expression in the novel and appetitive contexts, (C) but TMT only enhanced PL and IL c-Fos mRNA expression in the novel context. * – significant comparison for $p < .05$.

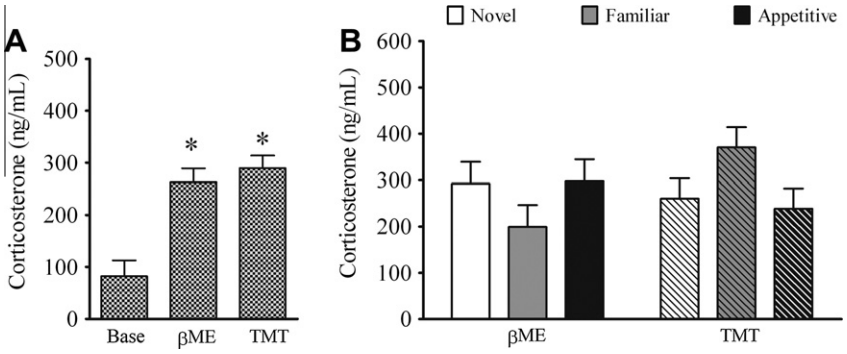

Fig. 4. TMT and β-ME presentation induced robust corticosterone responses that were not modulated by context. (A) Corticosterone responses induced by TMT and β-ME. (B) TMT and β-ME corticosterone responses in the novel, familiar, and appetitive contexts.

cage that was new to the animal, with new wood chips and a new lid – a context in which the animals had never been fed. The familiar context in this experiment thus differed from the novel context by being both familiar and "appetitive". In our design the testing context was never the home cage, but was a new context initially for all animals, which was made familiar by repeated exposure or made appetitive by repeated exposure that included food rewards. This design allowed a clearer differentiation between familiarity and appetitive expectancies. Both studies demonstrate that expression of unconditioned fear can depend on the context in which

unconditioned fear is induced, and, more specifically, that exposure to TMT in a context in which rewards have been received leads to enhanced fear expression. This would suggest that expectation of a reward in a context coupled with detection of threat in that context enhances unconditioned fear, but expectation of only a non-aversive outcome (i.e. safety) in a context coupled with detection of threat does not enhance unconditioned fear.

It should be noted that contextual and conditioned stimuli that have been explicitly paired with the absence of an unconditioned aversive stimulus (UCS) reduce conditioned fear expression (for examples see Charrier, Dangoumau, Puech, Hamon, & Thiebot, 1995; Stowell, Berntson, & Sarter, 2000; Yoon, Graham, & Kim, 2011). This may seem contradictory to the results of this study, however attenuated conditioned fear induced by presentation of safety signals and enhanced unconditioned fear induced by context-related expectancy mismatch, do not necessarily reflect the same mechanisms. In the presence of safety signals, conditioned fear responding is lowered, because the safety signal predicts the absence of the UCS. In this study, neither the familiar nor appetitive contexts had any predictive value about TMT presentation. Instead, TMT-induced freezing may have been enhanced in the appetitive context, because animals expected reward in this context, but instead detected threat. For context-related expectancy mismatch, we propose that conflict between expectation of a reward in the context and detection of threat in the same context enhances unconditioned fear.

TMT-induced freezing was enhanced in the appetitive context, but this was not associated with corresponding differences in corticosterone levels, although TMT exposure led to an overall corticosterone elevation. The relationship between fear expression and corticosterone release is not particularly consistent (Jellestad & Bakke, 1985; Selden, Everitt, & Robbins, 1991). For example, behavioral expression of conditioned fear can be disrupted by telencephalic depletion of norepinephrine with no impact on corticosterone responses induced by presentation of conditioned fear stimuli (Selden et al., 1991). Similarly, amygdala lesions decrease behavioral expression of contextual fear conditioning without impact on corticosterone responses induced by a contextual fear conditioned stimulus (Jellestad & Bakke, 1985). In our own data, exposure to β-ME induced a corticosterone response equivalent to that induced by TMT presentation without any corresponding expression of fear behavior (i.e. freezing). These findings further suggest that fear behavior and neuroendocrine stress responses are partly independent phenomena.

4.2. Enhanced TMT-induced freezing in the appetitive context is associated with enhanced c-Fos mRNA in the MeA, PL, and hippocampus

Presenting TMT in the novel context induced robust c-Fos mRNA expression in the PL and MeA. The MeA is critical for expression of TMT-induced freezing (Muller & Fendt, 2006), but the PL is critical for inhibiting TMT-induced freezing (Fitzpatrick et al., 2011). The PL may inhibit TMT-induced freezing by inhibiting neural activity in caudal fear systems that receive input from the PL (e.g. periaqueductal gray) (Fitzpatrick et al., 2011). Thus, the limited amount of TMT-induced freezing seen in the novel context may have reflected a balance between excitatory processes as a result of MeA activity and inhibitory processes as a result of PL activity. In the appetitive context, enhanced c-Fos mRNA expression was observed in the MeA, but not the PL. This more limited activation of PL activity with TMT presentation in the appetitive context may have resulted in reduced inhibition of caudal fear systems, allowing greater TMT-induced freezing to occur in this context. Thus, TMT-induced freezing in the appetitive context may have represented a combination of excitatory processes, as a result of MeA activity, and disinhibition of caudal fear systems, as a result of a failure to enhance PL activity. There was also a failure to enhance IL activity in the TMT/appetitive condition, but it is unlikely that this lack of IL effect contributed to enhanced TMT-induced freezing in the appetitive context, because directly inactivating the IL has no effect on TMT-induced freezing (Fitzpatrick et al., 2011).

Hippocampal c-Fos mRNA expression, especially in tasks where changes in context occur, can be indicative of contextual processing (Huff et al., 2006; Knapska & Maren, 2009; Milanovic et al., 1998; Rademacher, Napier, & Meredith, 2007; Strekalova et al., 2003). Enhanced hippocampal c-Fos mRNA expression was observed when TMT was presented in the novel and appetitive contexts, and these effects may be related to levels of contextual processing. Enhanced hippocampal activity in the TMT/novel condition may be critical for associating a threat stimulus with a context, whereas hippocampal activity in the TMT/appetitive condition may be critical for mediating context-related expectancy mismatch. In support of this assertion, previous research has demonstrated that hippocampal neural activity is critical for detecting discrepancies between an expected context and the currently experienced one (Mizumori et al., 1999).

TMT presentation did not induce c-Fos mRNA expression in the BNST, which is somewhat unexpected because a previous study has reported this effect (Day et al., 2004). However, the conditions under which TMT exposure occurred in the two studies were considerably different. Day et al. (2004) confined rats to small spaces, but in this study rats were exposed to TMT in an arena sufficiently large to observe freezing. These protocol differences may account for the observed differences in BNST c-Fos mRNA expression.

5. Conclusion

The results of this study suggest that unconditioned fear is enhanced in an appetitive context, perhaps as a result of context-related expectancy mismatch. The findings raise the possibility that expression of unconditioned fear is sensitive to contextual modulation, and this may be a salient difference between expression of conditioned and unconditioned fear. Further understanding of the neural basis of these differences, and other instances of modulated unconditioned fear responding (e.g. sensitization, dishabituation) (Staples, 2010), may deepen our neurobiological understanding of fear processing more generally. Given the salient role of contextual processing in abnormal instances of fear and anxiety (Gilbertson et al., 2007; Yamamoto et al., 2009), further research is needed to determine if context-related expectancy mismatch, and other instances of contextual modulation of fear, have any role to play in mediating abnormal instances of these emotions.

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GLUCOCORTICOID RECEPTORS AND EXTINCTION RETENTION DEFICITS IN THE SINGLE PROLONGED STRESS MODEL

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Abstract—Single prolonged stress (SPS) is a rodent model of post traumatic stress disorder that is comprised of serial application of restraint (r), forced swim (fs), and ether (eth) followed by a 7-day quiescent period. SPS induces extinction retention deficits and it is believed that these deficits are caused by the combined stressful effect of serial exposure to r, fs, and eth. However, this hypothesis remains untested. Neurobiological mechanisms by which SPS induces extinction retention deficits are unknown, but SPS enhances glucocorticoid receptor (GR) expression in the hippocampus, which is critical for contextual modulation of extinction retrieval. Upregulation of GRs in extinction circuits may be a mechanism by which SPS induces extinction retention deficits, but this hypothesis has not been examined. In this study, we systematically altered the stressors that constitute SPS (i.e. r, fs, eth), generating a number of partial SPS (p-SPS) groups, and observed the effects SPS and p-SPSs had on extinction retention and GR levels in the hippocampus and prefrontal cortex (PFC). PFC GRs were assayed, because regions of the PFC are critical for maintaining extinction. We predicted that only exposure to full SPS would result in extinction retention deficits and enhance hippocampal and PFC GR levels. Only exposure to full SPS induced extinction retention deficits. Hippocampal and PFC GR expression was enhanced by SPS and most p-SPSs, however hippocampal GR expression was significantly larger following the full SPS exposure than all other conditions. Our findings suggest that the combined stressful effect of serial exposure to r, fs, and eth results in extinction retention deficits. The results also suggest that simple enhancements in GR expression in the hippocampus and PFC are insufficient to result in extinction retention deficits, but raise the possibility that a threshold-enhancement in hippocampal GR expression contributes to SPS-induced extinction retention deficits.

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Abbreviations: Arp, actin related protein; ANOVA, analysis of variance; BB, blocking buffer; CP, changes in pixel; CS, conditioned stimulus; EI, extinction index; eth, ether; fs, forced swim; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; IL, infralimbic cortex; ISI, interstimulus; p-SPS, partial SPS; PFC, prefrontal cortex; PTSD, post traumatic stress disorder; r, restraint; SPS, single prolonged stress; SPSi, single prolonged stress with isoflurane instead of ether; UCS, unconditioned stimulus.

Key words: PTSD, fear, anxiety, extinction recall, contextual modulation, infralimbic cortex.

INTRODUCTION

Fear conditioning and extinction (a distinct learning process that occurs when a conditioned fear stimulus (CS) no longer predicts an aversive event (Bouton et al., 2006; Quirk et al., 2006)) have been used to understand the neurobiology of post traumatic stress disorder (PTSD). PTSD has been linked to deficits in the ability to maintain fear extinction (i.e. extinction retention deficit) (Hofmann, 2007; Milad et al., 2008, 2009; Hamm, 2009; Koenigs and Grafman, 2009; Norrholm et al., 2010), and it has been suggested that extinction retention deficits may follow exposure to traumatic stress (Milad et al., 2008) and may contribute to excessive fear/anxiety levels observed in PTSD (Quirk et al., 2006; Milad et al., 2008, 2009; Yamamoto et al., 2009). However, the specific neurobiological processes that lead to manifestation of extinction retention deficits in PTSD remain poorly understood.

Animal models of PTSD are useful for exploring neurobiological mechanisms that underlie specific PTSD symptoms (Armario et al., 2008; Yamamoto et al., 2009). While there are a number of available animal PTSD models (Armario et al., 2008), one particularly relevant model is single prolonged stress (SPS). SPS refers to serial application of restraint (r), forced swim (fs), and ether (eth) followed by a quiescent period of 7 days. SPS induces a number of effects that mimic PTSD symptoms, which include enhanced arousal (Khan and Liberzon, 2004; Kohda et al., 2007) and fast negative feedback of the hypothalamic–pituitary–adrenal (HPA) axis (Liberzon et al., 1997, 1999). SPS exposure also induces extinction retention deficits (Knox et al., 2012) and it is believed that the combined stressful effect of serial exposure to r, fs, and eth causes extinction retention deficits to manifest. However, it is also possible that exposure to only two or one of the single prolonged stressors (i.e. r, fs, eth) is sufficient to induce extinction retention deficits.

Neurobiological mechanisms by which exposure to SPS results in extinction retention deficits are unknown, but clues of such mechanisms are provided by the nature of extinction retention deficits induced by SPS. A previous report suggests that SPS induces extinction retention deficits without affecting conditioned fear memory (Knox et al., 2012). This suggests that SPS disrupts extinction retention by altering neural activity in

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brain regions specifically critical for extinction retention. Both the hippocampus and prefrontal cortex (PFC) are critical for extinction retention. The hippocampus is critical for contextual modulation of extinction retrieval (Corcoran and Maren, 2001, 2004; Bouton et al., 2006) and the infralimbic (IL) region of the PFC is critical for acquisition (Sierra-Mercado et al., 2006), consolidation (Sotres-Bayon et al., 2009), and retrieval (Lebron et al., 2004) of extinction memory. Thus, SPS-induced changes in neural function in the hippocampus and/or PFC might underlie SPS-induced extinction retention deficits. SPS enhances hippocampal GR expression and even though SPS has no effect on baseline or stress-enhanced corticosterone levels (Liberzon et al., 1997, 1999; Stout et al., 2010), increased GR expression might enhance glucocorticoid signaling, which can then have an impact on hippocampal function (McEwen, 2001; de Kloet et al., 2005) in such a manner so as to disrupt extinction retention. In support of this interpretation, both SPS-enhanced GR expression and SPS-induced extinction retention deficits are not observed immediately after exposure to the three SPS stressors (i.e. r, fs, eth), but manifest after a post-stress incubation period (i.e. full SPS model) (Liberzon et al., 1999; Knox et al., 2012). While this similar time line of symptom development suggests a link between SPS-enhanced GR expression and SPS-induced extinction retention deficits, this link has not been previously tested.

In this study, we examined whether the development of SPS-induced extinction retention deficits require the combined effect of serial exposure to r, fs, and eth. We also examined if SPS enhances GR levels in the PFC and if SPS-enhanced hippocampal and/or PFC GR expression contribute to SPS-induced extinction retention deficits. First, we compared extinction retention and GR levels in SPS animals to a control group. We then systematically examined extinction retention and GR levels in all possible combinations of two out of the three SPS stressors (partial SPS (p-SPS) groups) (Fig. 1C) and compared extinction retention and GR levels in these p-SPS animals to the previously mentioned control group. We did not examine single stressor exposure conditions simply because none of the two-stressor p-SPS groups produced extinction retention deficit, demonstrating that the combination of all three stressors was required. We also generated another p-SPS group by replacing the eth component of SPS with isoflurane (SPSi, Fig. 1B) and compared extinction retention and GR levels in SPSi animals to the same control group. In comparison to eth, brief exposure to isoflurane induces a smaller corticosterone response (Zardooz et al., 2010). It has been proposed that serial HPA axis activation, engaging psychological, physiological, and chemical pathways by the three SPS stressors, underlies the sensitizing effects of SPS on GR expression (Liberzon et al., 1999; Yamamoto et al., 2009). Thus, the inclusion of the SPSi group allowed for control of overall length of stress exposure, but with different chemical HPA axis activation.

EXPERIMENTAL PROCEDURES

Animals

Fifty-four adult male Sprague–Dawley rats (150 g upon arrival), obtained from Charles River (Portage, OR, USA) were used in this study. Upon arrival all rats were pair housed for a minimum of 3 days and then individually housed. All rats had *ad libitum* access to water and standard rat chow. Experimental procedures were approved by the Veteran Affairs Institutional Animal Care Usage Committee and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

SPS and p-SPSs

SPS ($n = 8$) consisted of 2 h of r immediately followed by 20 min of fs in a 55 L container. After a 15-min recuperation period, rats were exposed to eth in a desiccator until general anesthesia was induced (typically occurred within 5 min of eth exposure). p-SPSs were generated by reducing the number of stressors that comprise SPS or replacing the eth component of SPS with isoflurane (Fig. 1). The p-SPS groups generated in this study were r + fs ($n = 8$), r + eth ($n = 8$), fs + eth ($n = 7$), and SPSi ($n = 8$). All stress exposures were followed by a 7-day quiescent period, because both extinction retention deficits and enhanced GR expression within the SPS model require this post-stress incubation period to manifest (Liberzon et al., 1999; Knox et al., 2012). Rats assigned to the control group ($n = 8$) remained in the housing colony until fear conditioning commenced. In all experiments the effects of SPS and p-SPSs on extinction retention and GR expression were compared against a common control group.

Conditioned fear conditioning, extinction, and extinction retention

Fear conditioning, extinction, and extinction retention protocols were adopted from a previous study in which it was shown that SPS attenuates extinction retention (Knox et al., 2012). All behavioral procedures were conducted in observation chambers (MED Associates, St. Albans, VT, USA). The floor of each chamber consisted of a grid connected to a shock source (MED Associates), which delivered the footshock unconditioned stimulus (UCS, 1 mA, 1 s). A speaker mounted onto the wall generated a distinct auditory conditioned stimulus (CS, 10 s, 2 kHz, 80 dB). Cameras mounted onto the ceiling of the chambers were used to record videos, which were scored at a later date.

Two unique contexts were created by manipulating auditory, visual, and olfactory cues as previously described (Knox et al., 2012). Fear conditioning occurred in Context A and consisted of five paired presentations of the CS that co-terminated with the UCS. One day after conditioning, fear extinction occurred in Context B where rats were presented with 30 CSs in the absence of footshocks. One day after fear extinction, extinction testing commenced in Context B and consisted of 10 CS presentations. All sessions consisted of a baseline period of 180 s and interstimulus (ISI) interval of 60 s.

Western Blot procedure

One day after the extinction test all rats were euthanized by rapid decapitation, brains were removed and flash frozen in chilled isopentane, and then stored in a −80 °C freezer. For Western Blot, brains were thawed to −20 °C in a cryostat and the PFC dissected. The PFC was defined as all of the brain 4.77–2.2 mm anterior of Bregma (Paxinos and Watson, 1998), with

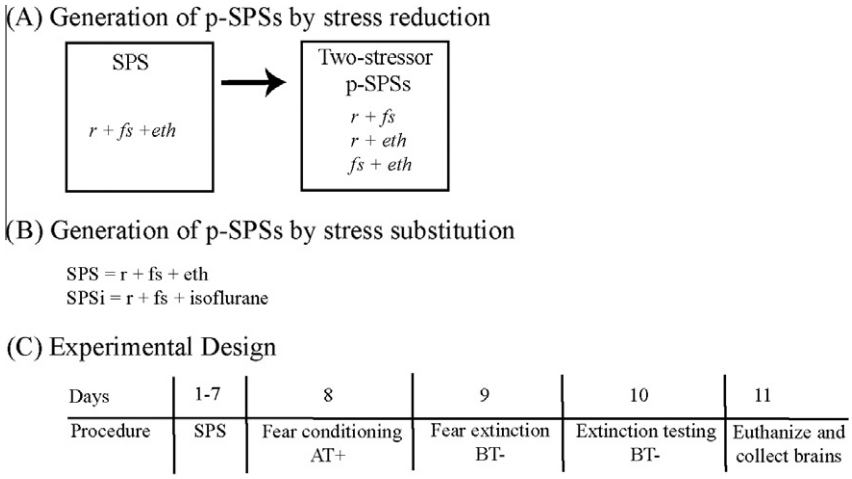

Fig. 1. Generation of p-SPSs by stress reduction and substitution, and experimental design. (A) Generation of p-SPSs by stress reduction. The first set of p-SPSs was generated by reducing the stressors that comprise SPS from three to two stressors. (B) Generation of p-SPSs by stress substitution. This p-SPS group was generated by substituting the eth component of SPS with isoflurane (SPSi). (C) General experimental design used in this study. The capital letter (A or B) represents the context in which a session took place, T represents the presence of tones, and + or − signs represent the absence or presence of footshocks, respectively. eth – ether, fs – forced swim, p-SPS – partial single prolonged stress, r – restraint.

the exception of the olfactory tubercles. After removal of the PFC, the cerebrum was separated from the brain stem, thawed on ice, and the hippocampus was removed. Brain samples were sonicated in homogenization buffer (50 mM Trizma base, 1 mM ethylenediaminetetraacetic acid, 10% sucrose, 4% sodium dodecyl sulfate, 2× protease inhibitor cocktail (Roche, USA), pH 7.0–7.4), centrifuged at 105,000*g* for 45 min, decanted, and protein content determined using a Pierce BCA Protein Assay Kit (Sigma–Aldrich, St. Louis, MO, USA). Approximately 20 μg of protein was then diluted into a 1× Lamelli sample buffer and stored in a −80 °C freezer until the Western Blot assay.

Assay of total GR protein levels (cytoplasm and nucleus) was adapted from Spencer et al. (2000). Samples were heated for 7 min at 70 °C and, along with a molecular weight (MW) ladder (Li-COR, Lincoln, NE, USA), electrophoresed on 7.5% Tris–HCl gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred onto nitrocellulose membranes. Membranes were then blocked in Tris buffer with 5% non-fat milk (i.e. blocking buffer (BB)). Nitrocellulose membranes were then probed for GR using a rabbit polyclonal GR antibody (Santa Cruz biotechnology Inc., Santa Cruz, CA, USA, M-20, 1:1000 in BB) overnight at 4 °C. After several washes in Tris buffer, membranes were incubated with an IRDye 800 conjugated anti-rabbit IgG secondary antibody (Li-COR, diluted 1:2000 in BB) for 1 h, rinsed, and then scanned using the Li-COR Odyssey Scanner for visualization of GR bands. After this, the nitrocellulose membranes that were previously probed for GR were then probed for actin related protein (Arp), which was used as a reference protein. Nitrocellulose membranes were probed for Arp with a rabbit polyclonal antibody (Santa Cruz Antibodies, Arp-2, 1:2000 in BB), and then incubated with the previously described secondary antibody (1:5000 in BB). Nitrocellulose membranes were then rinsed and scanned in the Li-COR Odyssey Scanner in order to visualize Arp bands.

Data and statistical analysis

Freezing was analyzed using Anymaze software (Stoelting Inc., Kiel, WI, USA). All videos were first recorded and stored on a digital hard-drive, and then converted into a divxx format. Divxx formatted videos were then imported into Anymaze for quantification of freezing behavior. Anymaze uses changes in pixel (CP) values in a video as a measure of movement. CPs are calculated and summed into a single score that varies from 0 to 100 at a rate of 10 times per second (i.e. 10 Hz). The program then uses two parameters to detect and quantify freezing behavior. An 'on-threshold' registers detection of freezing when CPs fall below a certain value (e.g. 70). An 'off-threshold' registers end of freezing behavior when CPs rise above a certain (e.g. 70). Thus, on-thresholds and off-thresholds control detection and quantification of freezing. Determination of the on (70) and off (75) thresholds used in this study was accomplished in a different set of animals (data not shown).

Freezing during CS presentation and corresponding ISI (e.g. CS1 and ISI1) was averaged into a single trial for all sessions and used as a measure of conditioned freezing. Freezing during fear conditioning was analyzed using a two factors design with the first factor being group (control, SPS or p-SPS) and the second factor being fear conditioning (baseline, trials 1–5). For fear extinction, freezing across three extinction trials were averaged into a block and analyzed using a similar two factors design with the second factor being fear extinction (baseline, blocks 1–10). Where appropriate, we also used trend analyses (e.g. quadratic and linear trend analyses) to make inferences about fear extinction. Extinction retention was analyzed in two different ways. First, freezing during the first five trials were split into two separate blocks and analyzed using a regular two factors design with the first factor being group and the second factor extinction test (baseline, block 1, block 2). This analysis allowed for better sensitivity of differences in extinction retention on early trials. Second, baseline freezing was subtracted from the average freezing score for the 10 CS-only trials to yield an extinction index (EI), which was then subjected to *t*-test (group vs. control). These indices have been previously used in other studies as sensitive and selective measure of extinction retention, because EIs are selective measures of CS-induced freezing (Corcoran and Maren, 2001, 2004; Knox et al., 2012), but because these EIs are also sensitive to baseline freezing levels, we performed a one-way analysis of variance (ANOVA) on baseline freezing levels during the extinction test, with the sole factor being group (control, SPS, r + fs, r + eth, fs + eth, SPSi). Post hoc comparisons and Bonferroni corrections were applied when appropriate.

Images of scanned nitrocellulose membranes were analyzed using Odyssey software (Li-COR). The integrated intensity (I.I.) of the GR and Arp bands were expressed as a ratio (GR/Arp) and used as a relative measure of GR expression. Relative

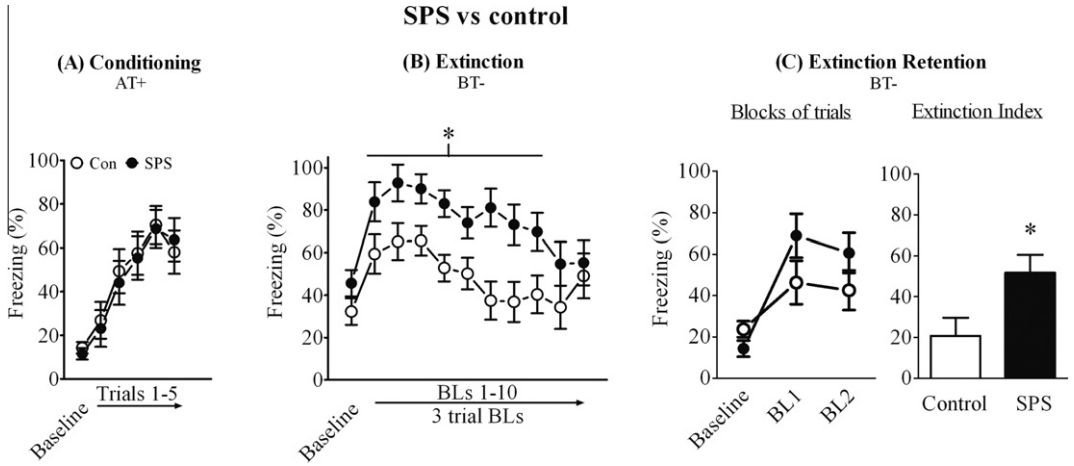

Fig. 2. The effects of SPS (i.e. r + fs + eth) on extinction retention. Controls from Fig. 1 are re-plotted on this graph. (A) SPS exposure had no effect on acquisition of conditioned fear, (B) enhanced expression of conditioned fear without affecting acquisition of extinction, and (C) induced deficits in extinction retention.

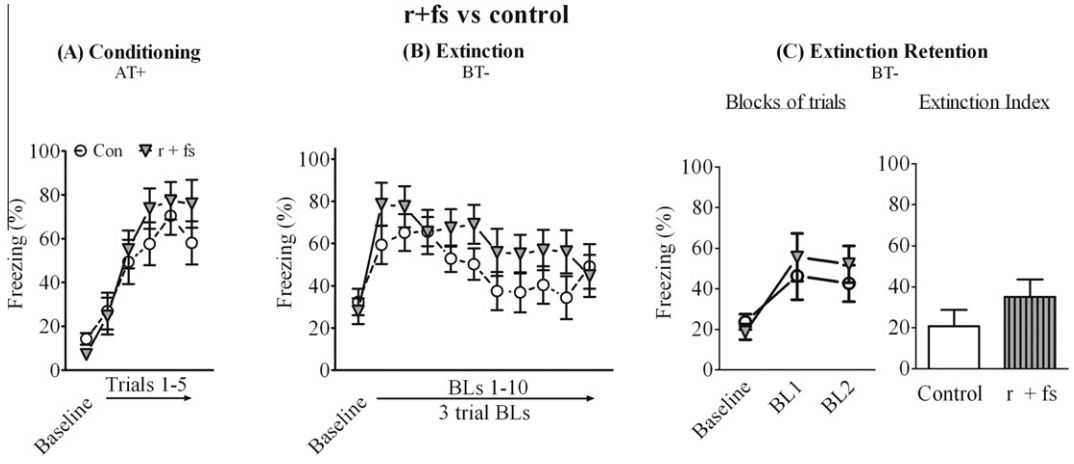

Fig. 3. The effects of r + fs on extinction retention. Controls from Fig. 1 are re-plotted on this graph. (A) Exposure to r + fs had no effect on acquisition of conditioned fear, (B) expression of conditioned fear, acquisition of extinction, or (C) extinction retention.

hippocampal and PFC GR levels were subjected to *t*-test (SPS or p-SPS vs. control), with Bonferroni corrections performed where necessary. For all statistical tests, the criterion for significance was set at $P < 0.05$.

RESULTS

SPS effects

In our first experiment we compared extinction retention between SPS and control animals. In all animals, fear-conditioned freezing increased across the fear-conditioning session ($F_{(5,70)} = 27.074$, $P < 0.001$), which suggested that all animals acquired fear conditioning and SPS did not alter this effect (group – $F_{(1,14)} = 0.032$, $P = 0.86$; group × fear conditioning – $F_{(5,70)} = 0.202$, $P = 0.96$, Fig. 2A). Conditioned freezing was enhanced at the start of the fear extinction session but decreased with CS presentations, which demonstrated expression of conditioned fear and acquisition of extinction, respectively (quadratic trend effect for fear extinction – $F_{(1,14)} = 6.918$, $P = 0.02$). SPS enhanced conditioned freezing ($F_{(1,14)} = 10.398$, $P = 0.006$), but during the last two blocks (or six CS presentations) of the extinction session, conditioned freezing was equivalent between SPS and control animals (block 9 – $t_{(14)} = 1.413$, $P = 0.18$; block 10 – $t_{(14)} = 0.412$, $P = 0.686$), suggesting that both control and SPS animals reached equivalent levels of extinction (Fig. 2B). During the extinction test, SPS animals demonstrated enhanced conditioned freezing as compared to controls, reflected in a group × extinction test interaction that approached significance ($F_{(2,28)} = 3.007$, $P = 0.066$), and a significant enhancement for EIs ($t_{(14)} = 2.466$, $P = 0.027$). These results are illustrated in Fig. 2C, are consistent with the assertion that SPS enhanced CS-induced freezing during the extinction retention session, and demonstrate that SPS disrupted extinction retention as previously reported (Knox et al., 2012).

p-SPS effects

Animals exposed to r + fs acquired fear conditioning ($F_{(5,70)} = 27.074$, $P < 0.001$) and acquisition of fear conditioning in these animals was not different to controls (group – $F_{(1,14)} = 0.43$, $P = 0.522$; group × fear

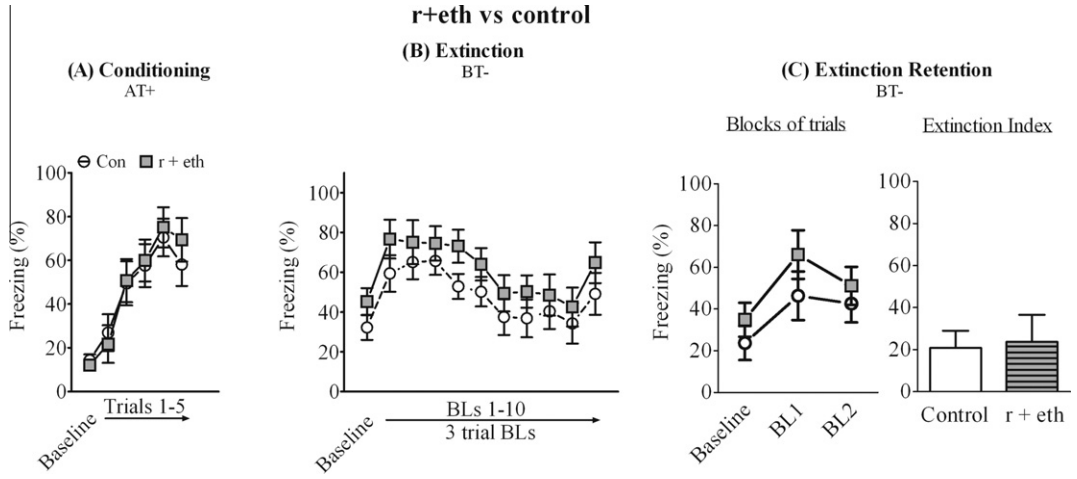

Fig. 4. The effects of r + eth on extinction retention. Controls from Fig. 1 are re-plotted on this graph. (A) Exposure to r + eth had no effect on acquisition of conditioned fear, (B) expression of conditioned fear, acquisition of extinction, or (C) extinction retention.

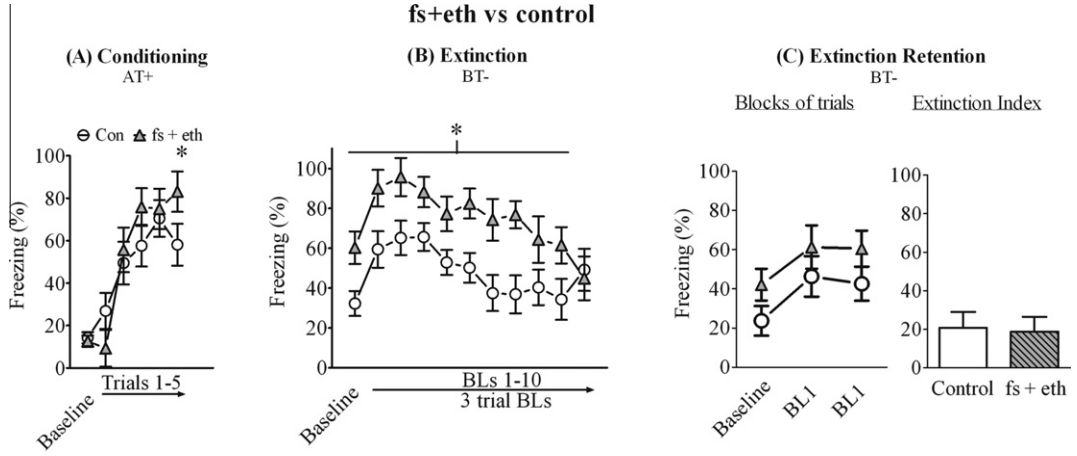

Fig. 5. The effects of fs + eth on extinction retention. Controls from Fig. 1 are re-plotted on this graph. (A) Exposure to fs + eth enhanced acquisition of fear and (B) expression of baseline and conditioned fear, but did not disrupt acquisition of extinction or (C) extinction retention.

conditioning – $F_{(5,70)} = 1.507$, $P = 0.199$, Fig. 3A). All rats expressed conditioned fear and acquired extinction (quadratic trend effect for fear extinction – $F_{(1,14)} = 8.235$, $P = 0.012$) and exposure to r + fs did not alter these effects (group – $F_{(1,14)} = 1.301$, $P = 0.273$; group × fear extinction – $F_{(10,140)} = 1.186$, $P = 0.306$, Fig. 3B). Exposure to r + fs had no effect on conditioned freezing during the extinction test (group – $F_{(1,14)} = 0.196$, $P = 0.665$; group × extinction test – $F_{(2.28)} = 0.869$, $P = 0.43$; EIs – $t_{(14)} = 1.212$, $P = 0.245$). These results are illustrated in Fig. 3C and suggest that exposure to r + fs had no effect on extinction retention.

Animals exposed to r + eth acquired fear conditioning (group – $F_{(5,70)} = 30.815$, $P < 0.001$) and acquisition of fear conditioning in these animals was not different from controls (group – $F_{(1,14)} = 0.044$, $P = 0.836$; group × fear conditioning – $F_{(5,70)} = 0.458$, $P = 0.806$, Fig. 4A). All rats expressed conditioned fear and acquired extinction (cubic trend effect for fear extinction – $F_{(1,14)} = 41.724$, $P < 0.001$), but exposure to r + eth had no effect on expression of conditioned fear or acquisition of extinction (group – $F_{(1,14)} = 1.913$, $P = 0.188$; group × fear extinction – $F_{(10,140)} = 0.163$, $P = 0.998$, Fig. 4B). Exposure to r + eth had no effect on conditioned freezing during the extinction test (group – $F_{(1,14)} = 1.576$, $P = 0.23$; group × extinction test – $F_{(2.28)} = 0.283$, $P = 0.755$; EIs – $t_{(14)} = 0.192$, $P = 0.851$). These results are illustrated in Fig. 4C and suggest that exposure to r + eth had no effect on extinction retention.

Animals exposed to fs + eth acquired fear conditioning ($F_{(5,65)} = 37.846$, $P < 0.001$) and acquisition of fear conditioning was enhanced in these animals relative to controls (main effect of group – $F_{(1,13)} = 6.457$, $P = 0.025$; Fig. 5A). All rats expressed conditioned fear and acquired extinction (quadratic trend effect for fear extinction – $F_{(1,13)} = 14.183$, $P = 0.002$). Exposure to fs + eth enhanced baseline and conditioned fear expression (group – $F_{(1,13)} = 10.283$, $P = 0.007$), however conditioned freezing during the last block of fear extinction was equivalent between controls and rats exposed to fs + eth ($t_{(13)} = 0.282$, $P = 0.783$), suggesting no disruption in acquisition of

extinction (Fig. 5B). Exposure to fs + eth had no effect on conditioned freezing during the extinction test (group – $F_{(1,13)} = 2.408$, $P = 0.145$; group × extinction test – $F_{(2.26)} = 0.046$, $P = 0.956$; EIs – $t_{(13)} = 0.177$, $P = 0.862$). These results are illustrated in Fig. 5C and suggest exposure to fs + eth had no effect on extinction retention.

SPSi effects

Next, we examined the effects of SPSi on extinction retention. Animals exposed to SPSi acquired fear conditioning ($F_{(5.65)} = 29.648$, $P < 0.001$) and acquisition of fear conditioning in these animals was not different to controls (group – $F_{(1,13)} = 0.072$, $P = 0.793$; group × fear conditioning – $F_{(5,65)} = 1.087$, $P = 0.376$; Fig. 6A). Expression of conditioned fear and acquisition of extinction was not affected by exposure to SPSi (cubic trend effect for fear extinction – $F_{(1,14)} = 16.299$, $P = 0.001$; group – $F_{(1,14)} = 0.448$, $P = 0.514$; group × fear extinction – $F_{(10,140)} = 0.91$, $P = 0.526$; Fig. 7B). Exposure to SPSi had no effect on conditioned freezing during the extinction test (group – $F_{(1,14)} = 0.353$, $P = 0.562$; group × extinction test – $F_{(2.28)} = 0.15$, $P = 0.861$; EIs – $t_{(14)} = 0.412$, $P = 0.686$). These results are illustrated in Fig. 6C and suggest that exposure to SPSi had no effect on extinction retention.

Baseline freezing during the extinction test

One-way ANOVA of baseline freezing for all stress groups during the extinction test was not significant ($F_{(5,41)} = 1.747$, $P = 0.146$), which suggests baseline freezing among the control and stress groups was not statistically different from each other. This is illustrated in Fig. 7.

Glucocorticoid receptors

Fig. 8A shows representative hippocampal GR and Arp bands from all groups in this study. SPS and all two-stressor p-SPSs enhanced GR expression in the hippocampus relative to controls (Table 1, Fig. 8B), but exposure to SPSi did not enhance GR expression relative to controls ($t_{(12)} = 0.13$, $P = 0.899$, Fig. 8C). Full SPS enhanced hippocampal GR expression relative to the two-stressor p-SPSs (Table 2, Fig. 8B) and SPSi ($t_{(11)} = 11.176$, $P < 0.001$, Fig. 7C). Fig. 9A shows representative PFC GR and Arp bands from all groups in this study. Exposure to SPS (Table 1, Fig. 9B), two-stressor p-SPSs (Table 1, Fig. 9B), and SPSi ($t_{(11)} = 11.176$, $P < 0.001$, Fig. 9C) enhanced GR expression in the PFC relative to controls. While the full SPS group had higher GR expression relative to the r + fs, r + eth (Table 2, Fig. 9B) and SPSi ($t_{(12)} = 5.51$, $P < 0.001$, Fig. 9C), SPS PFC GR enhancement was not different from the GR enhancement induced by exposure to fs + eth (Table 2, Fig. 9B).

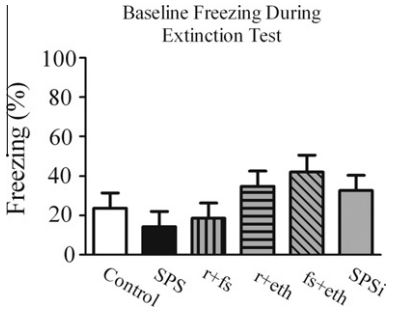

Fig. 7. The effects of SPS and p-SPSs on baseline freezing during the extinction test. Baseline freezing during the extinction test was not statistically among any of the stress groups.

DISCUSSION

In control animals, robust fear conditioning was observed and statistical analyses suggested that conditioned freezing decreased across the fear extinction session, which suggests acquisition of fear extinction. Furthermore, conditioned freezing during the extinction test was lower than conditioned freezing at the start of the fear extinction session, and this finding supports the assertion that fear extinction was acquired and retained in control animals. In comparison to controls, only exposure to the full three SPS stressors resulted in

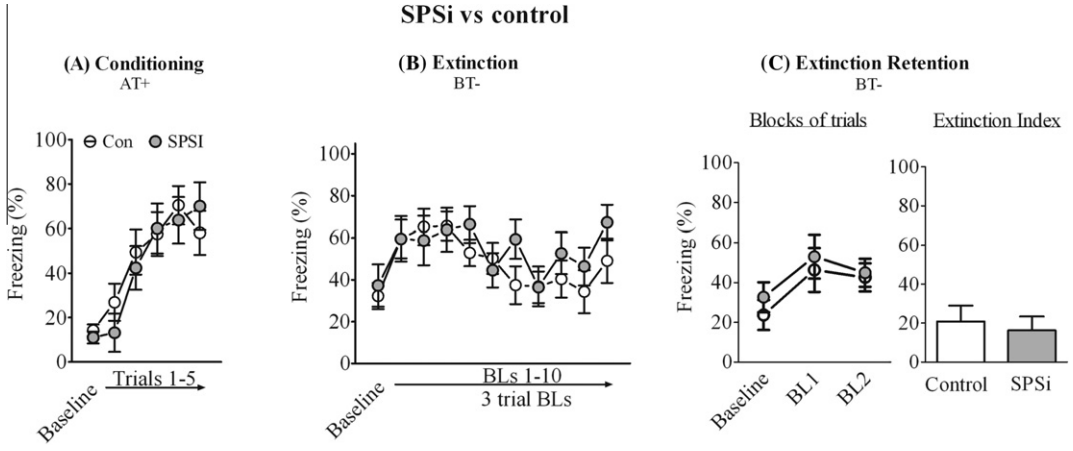

Fig. 6. The effects of SPSi (i.e. r + fs + isoflurane) on extinction retention. Controls from Fig. 1 are re-plotted on this graph. (A) Exposure to SPSi had no effect on acquisition of conditioned fear, (B) expression of conditioned fear, acquisition of extinction, and (C) extinction retention.

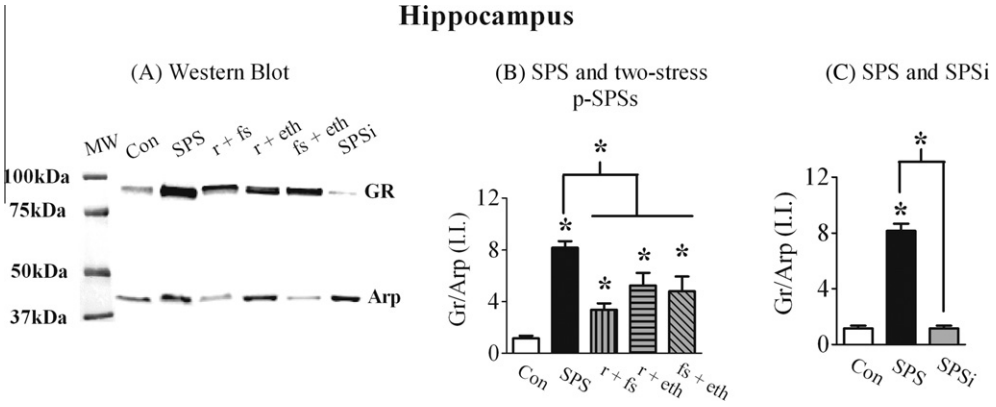

Fig. 8. The effects of exposure to SPS and p-SPSs on GR expression in the hippocampus. (A) Representative GR and Arp bands in the hippocampus. (B) SPS and all p-SPSs, except (C) SPSi, enhanced hippocampal GR expression relative to controls, but SPS induced maximal hippocampal GR expression. In (B) and (C), SPS and control bars are the same and are repeated for effective comparison to the two-stressor p-SPSs and SPSi. GR – glucocorticoid receptor, Arp – actin related protein.

Table 1. Planned comparisons using *t*-tests between SPS and control animals, and two-stressor p-SPSs and control animals revealed that all stress groups enhanced glucocorticoid receptor expression in the hippocampus and PFC

Comparison to control	Hippocampus	PFC
SPS	$t_{(10)} = 3.144$, $P = 0.01$	$t_{(10)} = 7.308$, $P < 0.001$
r + fs	$t_{(10)} = 4.219$, $P = 0.002$	$t_{(11)} = 3.171$, $P = 0.009$
r + eth	$t_{(12)} = 3.513$, $P = 0.004$	$t_{(12)} = 3.218$, $P = 0.007$
fs + eth	$t_{(11)} = 2.858$, $P = 0.016$	$t_{(11)} = 8.859$, $P < 0.001$

Planned comparisons using *t*-tests between SPS and control animals, and two-stressor p-SPSs and control animals revealed that all stress groups enhanced glucocorticoid receptor expression in the hippocampus and PFC.

Table 2. Post hoc comparisons revealed that SPS-induced maximal glucocorticoid receptor (GR) enhancement in the hippocampus in comparison to the other two-stressor p-SPSs. PFC GR enhancement induced by fs + eth was comparable to the enhancement induced by SPS. Where possible, stress groups that had similar means were averaged and compared to SPS in order to increase statistical power

Hippocampus		PFC	
SPS vs. r + fs	$t_{(10)} = 6.773$, $P < 0.001$	SPS vs. r + fs	$t_{(11)} = 3.868$, $P = 0.006$
SPS vs. (r + eth, fs + eth)	$t_{(11)} = 3.736$, $P = 0.012$	SPS vs. r + eth	$t_{(12)} = 3.558$, $P = 0.008$
		SPS vs. fs + eth	$t_{(12)} = 1.501$, $P = 0.159$

Post hoc comparisons revealed that SPS-induced maximal glucocorticoid receptor (GR) enhancement in the hippocampus in comparison to the other two-stressor p-SPSs. PFC GR enhancement induced by fs + eth was comparable to the enhancement induced by SPS. Where possible, stress groups that had similar means were averaged and compared to SPS in order to increase statistical power.

extinction retention deficits, which is consistent with the hypothesis that the combined effect of serial exposure to r, fs, and eth is required to induce SPS-like behavioral abnormalities. SPS did not disrupt acquisition of extinction, but did enhance conditioned fear memory expression during fear extinction, and this result is somewhat different from the results of a previous study (Knox et al., 2012). Given this finding, it can be asserted that SPS disrupts extinction retention by enhancing conditioned fear memory performance. If this is the case, SPS-enhanced freezing may not be consistently observed during fear conditioning due to ''ceiling effects'', i.e. high levels of freezing. Levels of conditioned freezing are typically lower during extinction testing, suggesting that SPS-enhanced conditioned fear is likely to manifest more reliably during extinction testing. While plausible, our current and prior observations do not necessarily support this interpretation. First, we did observe enhanced conditioned fear during fear extinction in one of our experimental groups (i.e. fs + eth) but this was not accompanied by extinction retention deficit. Second, in our previous experiments we have used conditioning procedures that yielded lower levels of conditioned freezing (45–70% maximal freezing), and we did not observe enhanced conditioned fear in SPS-exposed animals during fear conditioning and extinction (unpublished observation).

The finding that SPS and fs + eth enhanced conditioned fear performance during fear extinction, but r + fs and r + eth did not, could be interpreted to mean that the first part of SPS (i.e. r + fs) is not as critical for enhanced conditioned fear performance, and it is the second part of SPS (i.e. fs + eth) that is. This assertion is further supported by the finding that exposure to SPSi (i.e. r + fs + isoflurane) did not enhance conditioned

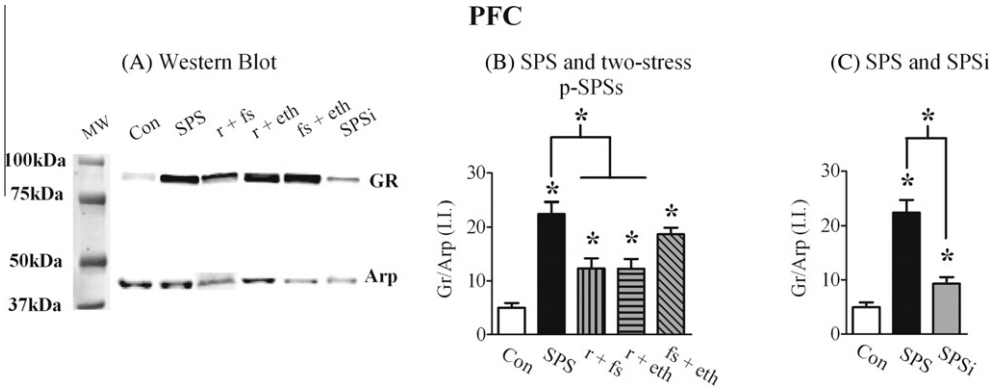

Fig. 9. The effects of exposure to SPS and p-SPSs on GR expression in the PFC. (A) Representative GR and Arp bands from PFC homogenates of all groups. (B, C) SPS and all p-SPSs enhanced PFC GR expression relative to controls. SPS induced the higher levels of PFC GR enhancement in comparison to r + fs, r + eth, and SPSi, but not in comparison to fs + eth. In (B) and (C), SPS and control bars are the same and are repeated for effective comparison to the two-stressor p-SPSs and SPSi.

fear memory performance. However, we have shown in three separate experiments that SPS does not enhance conditioned fear performance during fear extinction (Knox et al., 2012). In a number of pilot experiments in our laboratory using different conditioning parameters, we have also failed to consistently observe SPS-enhanced conditioned fear memory performance during fear extinction. Thus, enhanced conditioned fear performance during fear extinction induced by SPS exposure, and possibly exposure to fs + eth, does not appear to be a stable phenomenon.

Consistent with previous studies (Liberzon et al., 1999; Wang et al., 2009; Stout et al., 2010), the current results demonstrate that SPS enhanced GR expression in the hippocampus and extend these findings by demonstrating SPS enhances GR expression in the PFC. It is important to note that in these previous studies, SPS was not accompanied by footshock presentation, which suggests it is simply SPS exposure, not SPS and footshock exposure, that enhances GR expression. Even though exposure to p-SPS treatments did not lead to extinction retention deficits, exposure to most p-SPS treatments enhanced, to some degree, hippocampal and PFC GR expression relative to controls. This suggests that different combinations of two of the three SPS stressors (i.e. r, fs, eth) can induce various levels of enhancement in GR expression in the hippocampus and PFC. However, mere GR enhancements in these brain regions were insufficient to lead to extinction retention deficits. While exposure to r + fs, r + eth, and fs + eth enhanced hippocampal GR expression in comparison to controls, full SPS-enhanced hippocampal GR expression was significantly larger than all p-SPS groups. This could suggest that a threshold-enhancement in hippocampal GR expression contributes to extinction retention deficits induced by SPS. Because SPS exposure also enhanced PFC GR expression, it is also possible that a threshold-enhancement in hippocampal GR expression combined with enhanced PFC GR expression contributes to extinction retention deficits. Thus, while the results of this study suggest that GRs in extinction circuits contribute to extinction retention deficits in the SPS model, further research is needed to determine the exact role of GRs in extinction retention deficits in SPS.

Interestingly, replacing the eth component of SPS with isoflurane abolished extinction retention deficits, abolished enhanced hippocampal GR expression, and attenuated enhanced PFC GR expression within the SPS model. The hippocampal and PFC GR enhancement induced by r + fs was greater than the enhancement induced by SPSi (statistical analysis not shown), which suggests that exposure to isoflurane actively suppresses the sensitization of GRs induced by stress exposure. The mechanisms by which this suppressive effect occurs, and the significance of this suppression, remain to be determined.

A previous study has demonstrated that enhancing glucocorticoid signaling in the hippocampus induces contextual fear memory deficits that are reminiscent of contextual fear memory deficits in PTSD (Kaouane et al., 2012). How might a threshold-enhancement in hippocampal GR expression contribute to deficits in extinction retention in the SPS model? The results from a number of studies suggest that enhancing GR-corticosterone binding in the hippocampus enhances contextual memory formation (Pugh et al., 1997; Roozendaal and McGaugh, 1997; Donley et al., 2005; Gourley et al., 2009; Blundell et al., 2011). The memory for the context in which extinction is acquired primes extinction retrieval, and if extinction is tested in a context that is inconsistent with the extinction context, fear renewal occurs (Corcoran and Maren, 2001, 2004; Bouton et al., 2006). A threshold-enhancement in hippocampal GRs in SPS animals may enhance GR-corticosterone binding in the hippocampus during fear extinction, which would enhance memory for the extinction context. If this occurred, then SPS animals may have an enhanced ability to detect contextual inconsistencies between fear extinction and extinction testing, which would make SPS animals more likely to show fear renewal. In this study, animals were tested for extinction in the same physical space that extinction was acquired (i.e. context B), but the passage of time

can serve as a change in contextual feature (Bouton et al., 2006; Monfils et al., 2009) and contribute to fear renewal-like effects (Bouton et al., 2006). In this study, there was a 24-h interval between fear extinction and extinction testing, which is standard in most fear extinction experiments. This 24-h interval may have served as a temporal contextual inconsistency for SPS animals, but not control animals, and resulted in a fear renewal-like effect in SPS animals during extinction testing. This hypothesis is speculative, but empirical data support aspects of this hypothesis. For example, previous studies have shown that contextual memory performance is enhanced in SPS animals (Imanaka et al., 2006; Iwamoto et al., 2007; Kohda et al., 2007), and this enhancement is dependent on increases in hippocampal GR expression (Kohda et al., 2007). Also, SPS animals show enhanced conditioned fear expression when extinction is tested in a context that has inconsistent physical features to the extinction context (Knox et al., 2012), which could be interpreted to mean that SPS animals are more sensitive to contextual inconsistencies between extinction training and testing. Thus, a SPS-induced threshold-enhancement in hippocampal GR expression could lead to enhanced memory for an extinction context, which enhances contextual feature discrimination between fear extinction and extinction testing. In turn, this would facilitate fear renewal-like effects. The PFC is also critical for contextual processing (Gilmartin and Helmstetter, 2010; Li et al., 2011; Orsini et al., 2011), which raises the possibility that SPS-enhanced GR expression in the PFC might also contribute to enhanced context formation during fear extinction, which then results in enhanced contextual feature discrimination between fear extinction and extinction testing. In support of this assertion, the results from a previous study demonstrate that neonatal treatments that enhance GR expression in the mPFC also result in extinction retention deficits (Wilber et al., 2009). However, it should be noted that the methods used in Wilber et al. (2009) did not allow for a differentiation between extinction retention deficits induced by deficits in extinction consolidation vs. changes in contextual modulation of extinction. While the above stated hypotheses are plausible, further research is needed to examine them.

There are other neurobiological mechanisms that could explain SPS-induced extinction retention deficits. SPS attenuates excitatory tone in the mPFC (Knox et al., 2010), and if this applied to the IL, then this may disrupt extinction consolidation and/or retrieval; both processes that are dependent on the IL (Lebron et al., 2004; Sotres-Bayon et al., 2009). The amygdala is critical for expression of conditioned fear (Maren, 2001; Pare et al., 2004; Bouton et al., 2006), and IL cortical control of amygdala regions is critical for extinction retention (Rosenkranz and Grace, 2002; Rosenkranz et al., 2003; Pare et al., 2004; Quirk and Mueller, 2008). Furthermore, sensitizing amygdala neural activity can result in extinction retention deficits (Rau et al., 2005). In previous studies, we reported that SPS exposure did not enhance conditioned fear expression (Knox et al., 2012) or alter inhibitory and excitatory tone in the amygdala (Knox et al., 2010). This suggests that SPS does not affect extinction retention by altering amygdala function. In this study, however, SPS increased conditioned fear expression during fear extinction, which raises the possibility that SPS might disrupt extinction retention by altering amygdala function. Further research is needed to examine these possibilities.

Exposure to other types of stressors results in extinction retention deficits, although these protocols have different effects on GR expression. Chronic stress exposure decreases GR expression in the hippocampus (Mizoguchi et al., 2003) and induces extinction retention deficits (Miracle et al., 2006; Baran et al., 2009; Green et al., 2011; Wilber et al., 2011). If chronic stress-induced changes in hippocampal GRs underlie chronic stress-induced extinction retention deficits, then it would appear that a threshold-enhancement and simple decreases in hippocampal GR expression can have the same effect on extinction retention. This apparent inconsistency demonstrates that a singular mechanism does not underlie all stress-induced deficits in extinction retention. As a result, further research is needed to clarify the different neurobiological mechanisms by which stress exposure can result in extinction retention deficits.

CONCLUSION

The results of this study demonstrate that the combined stressful effect of serial exposure to r, fs, and eth results in extinction retention deficits. The results also demonstrate that SPS enhances GR expression in the PFC. While the results of the study do not support the hypothesis that a simple enhancement in hippocampal or PFC GR levels is sufficient for extinction retention deficits, the results raise the possibility that a threshold-enhancement in hippocampal GR expression is required, potentially with combined PFC GR upregulation, for SPS-induced extinction retention deficits to manifest.

CONFLICT OF INTEREST STATEMENT

Dr. Dayan Knox, Tori Nault, Curtis Henderson and Dr. Israel Liberzon have no conflict of interest concerning the findings presented in this manuscript.

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GLOSSARY

Extinction retention: The ability to retain and express an extinction memory after it has been acquired

Fear conditioning: Acquisition of a memory that involves an association of a neural stimulus with an unconditioned stimulus

Fear extinction: Acquisition of a memory that involves an association of previously fear-conditioned stimulus with a non-aversive outcome

Fear renewal: The process by which extinction retrieval is suppressed and conditioned fear renewed, because extinction is tested in a context that is different to the context in which extinction was learned

Post traumatic stress disorder: An anxiety disorder brought on by exposure to a traumatic and/or stressful event

Single prolonged stress: Serial exposure to 2 h of restraint, 20 min of forced swim, and ether exposure followed by a quiescent period of at least 7 days

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